Alert

Peripheral Vascular Disease

What is peripheral vascular disease?

This refers to diseases of blood vessels outside the heart and brain. It's often a narrowing of vessels that carry blood to the legs, arms, stomach or kidneys. There are two types of these circulation disorders:

- **Functional** peripheral vascular diseases don't have an organic cause. They don't involve defects in blood vessels' structure. They're usually short-term effects related to "spasm" that may come and go. Raynaud's disease is an example. It can be triggered by cold temperatures, emotional stress, working with vibrating machinery or smoking.
- **Organic** peripheral vascular diseases are caused by structural changes in the blood vessels, such as inflammation and tissue damage. Peripheral artery disease is an example. It's caused by fatty buildups in arteries that block normal blood flow.

What is peripheral artery disease?

Peripheral artery disease (PAD) is a condition similar to coronary artery disease and carotid artery disease. In PAD, fatty deposits build up in the inner linings of the artery walls. These blockages restrict blood circulation, mainly in arteries leading to the kidneys, stomach, arms, legs and feet. In its early stages a common symptom is cramping or fatigue in the legs and buttocks during activity. Such cramping subsides when the person stands still. This is called "intermittent claudication." People with PAD often have fatty buildup in the arteries of the heart and brain. Because of this association, most people with PAD have a higher risk of death from heart attack and stroke.

How is peripheral artery disease diagnosed and treated?

Techniques used to diagnose PAD include a medical history, physical exam, ultrasound, X-ray angiography and magnetic resonance imaging angiography (MRA).

Most people with PAD can be treated with lifestyle changes, medications or both. Lifestyle changes to lower your risk include:

- Stop smoking (smokers have a particularly strong risk of PAD).
- Control diabetes.
- Control blood pressure.
- Be physically active (including a supervised exercise program).
- Eat a low-saturated-fat, low-cholesterol diet.

PAD may require drug treatment, too. Drugs include:

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DID YOU KNOW?

Of the 12 million people who are affected by peripheral artery disease (PAD) nearly 75% will never experience symptoms. Women are less likely to have symptoms than men.

read more...

On average, smokers experience symptoms of peripheral artery disease (PAD) 10 years earlier than non-smokers.

learn more...

- medicines to help improve walking distance (cilostazol and pentoxifylline).
- antiplatelet agents.
- cholesterol-lowering agents (statins).

In a minority of patients, lifestyle modifications alone aren't sufficient. In these cases, angioplasty or surgery may be necessary.

Angioplasty is a non-surgical procedure that can be used to dilate (widen) narrowed or blocked peripheral arteries. A thin tube called a catheter with a deflated balloon on its tip is passed into the narrowed artery segment. The balloon is then inflated, compressing the plaque and dilating the narrowed artery so that blood can flow more easily. Then the balloon is deflated and the catheter is withdrawn.

Often a stent — a cylindrical, wire mesh tube — is placed in the narrowed artery with a catheter. There the stent expands and locks open. It stays in that spot, keeping the diseased artery open.

If the narrowing involves a long portion of an artery, surgery may be necessary. A vein from another part of the body or a synthetic blood vessel is used. It's attached above and below the blocked area to detour blood around the blocked spot.

See the Related Items box above for links to the **Cardiology Patient Page** in *Circulation, Journal of the American Heart Association*:

- Diseases of the Veins

Related AHA publications:

- Heart and Stroke Facts
- Heart Disease and Stroke Statistics Update

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Peripheral Vascular Disease

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Angioplasty and Cardiac Revascularization Treatments and Statistics
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Angioplasty, Percutaneous Transluminal Coronary (PTCA)
Anticoagulants
Atherectomy
Atherosclerosis
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Heart Damage Detection
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Cerebral Circulation

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Definition

Cerebral circulation, the supply of blood to the brain

Understanding how the brain is supplied with blood is important because a significant number of neurological disorders that result in hospital admissions are due to problems with cerebral vascular disease. In some hospitals, nearly half the admissions due to neurologic disorders relate in some form to problems with cerebral circulation.

Insufficient supply of blood to the brain can cause fainting (syncope) or a more severe loss of consciousness. A continuous supply of highly oxygenated blood is critical to brain tissue function and a decrease in pressure or oxygenation (percentage of oxygen content) can cause tissue damage within minutes. Depending on a number of other physiological factors (e.g., temperature, etc.), brain damage or death may occur within two to 10...

[The entire page is 1818 words long]

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subarachnoid space

The space between the arachnoidea and pia mater, traversed by delicate fibrous trabeculae and filled with cerebrospinal fluid. Since the pia mater immediately adheres to the surface of the brain and spinal cord, the space is greatly widened wherever the brain surface exhibits a deep depression (for example, between the cerebellum and medulla); such widenings are called cisternae. The large blood vessels supplying the brain and spinal cord lie in the subarachnoid space.

Synonym: cavum subarachnoideum, subarachnoid cavity.

(05 Mar 2000)

Previous: subarachnoid anaesthesia, subarachnoid cavity, subarachnoid haemorrhage
Next: subarcuate, subarcuate fossa, subareolar

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Cerebral Circulation, Poor
Signs, symptoms, indicators
Contributing risk factors
Treatment recommendations

Poor Cerebral Circulation

Health problems rarely occur in isolation or for obvious reasons

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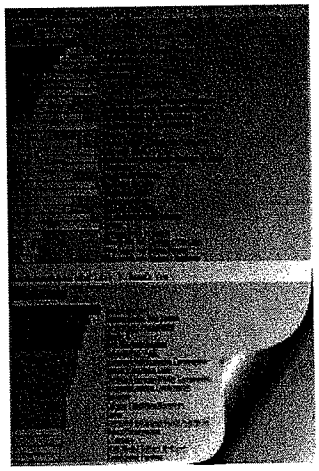

Cerebral circulation involves a most remarkable system of supply and demand. The brain, having a cell metabolism utterly dependent on immediate availability of oxygen, and having by far the highest metabolic rate of any organ in the body, requires excellent circulatory flow in order to function.

Please note that it is extremely important to obtain an accurate diagnosis before trying to find a cure. Many diseases and conditions share common symptoms: if you treat yourself for the wrong illness or a specific symptom of a complex disease, you may delay legitimate treatment of a serious underlying problem. In other words, the greatest danger in self-treatment may be self-diagnosis. If you do not know what you really have, you can not treat it!

Knowing how difficult it is to weed out misinformation and piece together countless facts in order to see the "big picture", we now provide simple online access to The Analyst™. Used by doctors and patients alike, The Analyst™ is a computerized diagnostic tool that sits on a vast accumulation of knowledge and research. By combining thousands of connections between signs, symptoms, risk factors, conditions and treatments, The Analyst™ will help to build an accurate picture of your current health status, the risks you are running and courses of action (including appropriate lab testing) that should be considered. Full information is available here.

The brain is the most active metabolic organ in the body, and also one of the most vulnerable to metabolic upset. Without significant variation between wakefulness or sleep or levels of physical/mental activity, the central nervous system uses some 15-20% of one's oxygen intake and only a slightly lesser percentage of the heart's output. Circulating blood volume within the brain at any instant is about 750ml and remains essentially constant, although regional variations occur within the

brain with change in mental activity.

Virtually all of this oxygen use is for conversion of glucose to CO2. Since neural tissue has no mechanism for storage of oxygen, there is an oxygen metabolic reserve of only about 8-10 seconds.

When brain cells are deprived of their blood supply, a stroke occurs. Without access to vital nutrients and oxygen, brain cells die. The effects of a stroke can vary widely depending on where it occurs in the brain, the severity of the attack, and the general health of the person. A minor stroke may not even be noticed, while a major one can cause crippling mental and physical disabilities or even death.

The brain automatically regulates the blood pressure between a range of about 50 to 140mm Hg. If pressure falls below 50mm Hg, adjustments to the vessel system cannot compensate, brain perfusion pressure also falls, and the result may be hypoxia and circulatory blockage. Pressure elevated above 140mm Hg results in increased resistance to flow in the cerebral arterial tree. Excessive pressure can overwhelm resistance, leading to elevated capillary pressure, loss of fluid to the meager tissue compartment, and brain swelling.

Causes & Development

Disruptions to the brain's blood circulation include cerebral hemorrhage, thrombosis, embolism, or subarachnoid hemorrhage. These usually result from pre-existing vascular disease or congenital weakness and may be precipitated by trauma. Most commonly these problems occur in older persons.

- **Hemorrhage** usually occurs in atherosclerotic vessels. The resulting blood clot destroys brain tissue, and the neural tissue remaining next to the clot may be softened, leading to later complications. The clot and dead tissue are removed by macrophages, and the damaged area is invaded by connective tissue and glia, often producing a fluid-filled cyst.

- **Thrombosis** most commonly involves formation of a clot at a site of vessel lumen constriction due to growth of atherosclerotic plates. Blockage of circulation leads to tissue softening and death, and to congestion of flow and edema in adjacent areas.

- **Embolism** is the blockage of a cerebral vessel by a physical object, such as a dislodged clot, air, tumor cells, infectous mass. Often the situation involves multiple embolisms, complicating the clinical picture.

- **Aneurisms** are the expansion of a vessel, usually an artery, and these balloons may reach a diameter of several inches. If this occurs within the cranial cavity, the displacement of neural tissue and the compression of other vessels and of cranial nerves can obviously lead to severe problems. Most arise from the middle cerebral artery or the internal carotid, and if expansion is slow enough, there may be extensive erosion of bone. Clinically there is the added danger that the weakened vessel will rupture, giving the added problems of subarachnoid hemorrhage. Treatment usually involves surgery, if the vessel is accessible. Methods include removal of the sac or reinforcing the vessel wall with muscle or connective tissue, or ensheathing or replacing the weakened vessel with plastic.

Chronic hypertension may result in vascular sclerotic lesions and lead to headache, dizziness, digestive symptoms, and even seizures. The final outcomes may include any of the problems described above.

Treatment & Prevention

Treatment involves weight loss (if this is part of the problem), sodium restriction, and drugs to reduce pressure, in addition to symptomatic treatments if these do not subside.

Complications

Part of the clinical problem is that by the time these symptoms manifest themselves, there may have been extensive damage.

Signs, symptoms & indicators of Poor Cerebral Circulation:

Symptoms - Mind - General	**Short-term memory failure**
	Reduced/poor mental clarity

Risk factors for Poor Cerebral Circulation:

Circulation	**Hypertension**

Recommendations and treatments for Poor Cerebral Circulation:

Botanical	**Ginkgo Biloba**
	Vinpocetine

KEY

Weak or unproven link
Strong or generally accepted link
Likely to help

GLOSSARY

Arterial (Arteries, Artery)
Blood that leaves the heart. When it leaves the right ventricle, it is venous blood; and when it leaves the left ventricle, through the aorta, it is fresh and oxygenated. After it has passed out to the capillaries and started to return, it is venous blood.

Capillary
Any of the smallest blood vessels connecting arterioles with venules and forming networks throughout the body.

Central Nervous System (CNS)
A collective term for the brain, spinal cord, their nerves, and the sensory end organs. More broadly, this can even include the

neurotransmitting hormones instigated by the CNS that control the chemical nervous system, the endocrine glands.

Chronic (Chronicity)
Usually referring to chronic illness: Illness extending over a long period of time.

Cysts (Cyst)
A closed pocket or pouch of tissue; a cyst may form within any tissue in the body and can be filled with air, fluid, pus, or other material. Cysts within the lung generally are air-filled, while cysts involving the lymph system or kidneys are fluid filled. Cysts under the skin are benign, extremely common, movable lumps. These may develop as a result of infection, clogging of sebaceous glands, developmental abnormalities or around foreign bodies.

Edema
Abnormal accumulation of fluids within tissues resulting in swelling.

Embolism
Obstruction of a vessel by an abnormal body, usually a detached blood clot.

Glucose
A sugar that is the simplest form of carbohydrate. It is commonly referred to as blood sugar. The body breaks down carbohydrates in foods into glucose, which serves as the primary fuel for the muscles and the brain.

Hemorrhage (Hemorrhaging)
Profuse blood flow.

Hg
The chemical symbol for mercury, often used to indicate pressure measurements in either inches or millimeters.

Hypertension
High blood pressure. Hypertension increases the risk of heart attack, stroke, and kidney failure because it adds to the workload of the heart, causing it to enlarge and, over time, to weaken; in addition, it may damage the walls of the arteries.

Lesion (Lesions)
Any damage to tissue structure or function; an abnormal change in body tissue caused by disease or injury. A scar is a lesion, as is cancer, a stomach ulcer or a pimple.

Lumen
Space in the interior of a tubular structure.

Macrophage (Macrophages)
An immune system cell that scavenges bacterial and other foreign material in the blood and tissues. It is a mature form of what is released from the marrow as a monocyte. A macrophage lives long, can digest much detritus, and is able to wear particles of odd food on its outer membrane. This allows T-cell and B-cell lymphocytes to taste the particle (an epitope) and form an antibody response. Further, these macrophages, traveling as monocytes, will take up permanent residence in many tissues, providing them with immunity. They line the spleen, form the cleansing Kupffer cells in the liver, make up the "dust cells" that protect the lungs, protect the synovial fluids of the joints, and form the microglial cells that provide protection to the brain and nerve tissues. Essentially the macrophages clean up messes and act as the intermediates between innate and acquired immunity.

Metabolism (Metabolic, Metabolize, Metabolizes, Metabolizing)
The chemical processes of living cells in which energy is produced in order to replace and repair tissues and maintain a healthy body. Responsible for the production of energy, biosynthesis of important substances, and degradation of various compounds. Also defined as the sum total of changes in an organism in order to achieve a balance (homeostasis): Catabolic burns up, anabolic stores and builds up; the sum of their work is metabolism.

Milliliter (mL)
0.001 or one thousandth of a liter.

Millimeter (Millimeters, mm)
A metric unit of length equaling one thousandth of a meter, or one tenth of a centimeter. There are 25.4 millimeters in one inch.

Perfusion
Usually Coronary/Myocardial perfusion: Flow of blood to the heart and/or blood vessels surrounding the heart.

Seizure (Seizures)
While there are over 40 types of seizure, most are classed as either **partial seizures** which occur when the excessive electrical activity in the brain is limited to one area or **generalized seizures** which occur when the excessive electrical activity in the brain encompasses the entire organ. Although there is a wide range of signs, they mainly include such things as falling to the ground; muscle stiffening; jerking and twitching; loss of consciousness; an empty stare; rapid chewing/blinking/breathing. Usually lasting from between a couple of seconds and several minutes, recovery may be immediate or take up to several days.

Sodium
An essential mineral that our bodies regulate and conserve. Excess sodium retention increases the fluid volume (edema) and low sodium leads to less fluid and relative dehydration. The adult body averages a total content of over 100 grams of sodium, of which a surprising one-third is in bone. A small amount of sodium does get into cell interiors, but this represents only about ten percent of the body content. The remaining 57 percent or so of the body sodium content is in the fluid immediately surrounding the cells, where it is the major cation (positive ion). The role of sodium in the extracellular fluid is maintaining osmotic equilibrium (the proper difference in ions dissolved in the fluids inside and outside the cell) and extracellular fluid volume. Sodium is also involved in nerve impulse transmission, muscle tone and nutrient transport. All of these functions are interrelated with potassium.

Stroke (Stroke-Type Event)
A sudden loss of brain function caused by a blockage or rupture of a blood vessel that supplies the brain, characterized by loss of muscular control, complete or partial loss of sensation or consciousness, dizziness, slurred speech, or other symptoms that vary with the extent and severity of the damage to the brain. The most common manifestation is some degree of paralysis, but small strokes may occur without symptoms. Usually caused by arteriosclerosis, it often results in brain damage.

Thrombosis
Formation of blood clots causing vascular obstruction.

Vascular
Relating to the blood vessels of the body. The blood vessels of the body, as a group, are referred to as the vascular system. They are composed of arteries, veins and capillaries - arteries that pass oxygen-rich blood to the tissues of the body; veins which return oxygen-depleted blood from the tissues to the lungs for oxygen; and the capillaries that are the tiniest vessels and are between the arteries and veins.

Last updated: Feb 29, 2008

Poor Circulation (Peripheral Vascular Disease)

Information for health professionals on peripheral vascular disease

Peripheral vascular disease is the medical name given to a group of problems that causes poor circulation to the feet and legs. The most common cause of this is artherosclerosis ("hardening of the arteries") in which there is a gradual thickening and hardening of the walls of the arteries (the blood vessels that bring blood to the extremities from the heart). Diabetes is the most common cause of peripheral vascular disease.

Symptoms of poor circulation:

The symptoms that are experienced can depend on which artery is affected and how much the blood flow is reduced.

Some of the symptoms include:
* Claudication (this is a dull cramping pain in the calf muscle that comes on after walking a certain distance - it is relieved by rest).
* Numbness or tingling in the foot, or toes can occur.
* Changes in the color of the skin (it becomes more pale, bluish, or reddish).
* Changes in skin temperature (the foot becomes cooler. See cold feet).
* Skin breakdowns, infection and sores do not heal as well as they should.

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What causes poor circulation to the foot:

Poor circulation is most commonly caused by a progressive blocking of the arteries in the leg (athersclerosis). Those with diabetes are more likely to develop poor circulation to the foot. Other risk factors for developing poor circulation include a lack of physical activity,smoking, high blood pressure and high cholesterol.

The effects of poor circulation (peripheral vascular disease) on the foot:

The biggest affect of poor circulation on the foot, is that its problems (such as sores, infections, cuts, etc) develop, they do not heal as well as they

should. In many cases they do not heal at all without special care. The reason for this is that the blood carries vital elements (eg oxygen) that the bodies tissues need for vitality and healing.

Treatment of poor circulation:

Poor circulation can be treated by your doctor or vascular surgeon in a number of ways:
* Good control of the blood glucose level is very important if diabetes is present.
* Other risk factors - such as lack of exercise; high blood pressure; smoking; and high cholesterol levels also need to be addressed.
* Drugs can be used to prevent the blood clotting (antiplatelet agents and anticoagulants).
* Angioplasty can be used to enlarge the narrowed peripheral arteries.
* A vein from another part of the body can be used to bypass the narrow or blocked artery can be used by the vascular surgeon.

Why foot care is important for those with poor circulation to the foot (peripheral vascular disease):

As the healing from skin breakdowns, sores and cuts on the foot is poor due to the poor circulation, extra special care is needed to prevent problems from developing as well as care of any problems that develop. This care includes:
* Preventing trauma and accidents (eg wearing shoes to prevent trauma)
* Wearing footwear that is well fitted and not causing any pressure areas.
* Seeking professional help from a podiatrist for the cutting of toe nail (any accidents from self care here may prove to be costly)
* **Corns and calluses** need treatment. If they are allowed to progress the skin may break down and sores may develop beneath them and prove difficult to heal.

Podiatric management of those with poor circulation to the foot (peripheral vascular disease):

Podiatric management of those with peripheral vascular disease (poor circulation) should include:
* A complete evaluation and assessment of the status of the circulation to the foot and communication to you about the risk that the foot is at for complications developing.
* Periodic reassessment of this status.
*Advice on foot care and **fitting footwear.**
*Care of toenail, **corns, calluses** and other foot conditions.
* Management of any wounds, sores, infections that may develop as the result of poor circulation.

Self management for those with poor circulation to the foot (peripheral vascular disease):

There is a lot you can do to help yourself if you have poor circulation. Follow your doctors advice (especially about exercise) and take advice from a **Podiatrist** about foot care and **footwear fitting. Foot care** for those with diabetes it is extremely important.

"I went to a Podiatrist and they cut me. I will never go back"

"That is unfortunate and can happen by accident on rare occasions as Podiatrists use sharp instruments to cut toenails and reduce corns and callus. It would not have happened deliberately. This has to be weighed against the high risk that you are at for the skin breaking down (sores developing) if professional care is not received."

Links of relevance to poor circulation (peripheral vascular disease):

Cold feet
Chilblains
Diabetes foot care
Find a Podiatrist
ePodiatry's resources on peripheral vascular disease
Books on foot care
Ask a question in the foot health forum about poor circulation

Buy footcare products:

USA & Canada:

UK & Europe:

Foothealthcare.com

Australia & NZ:

Coming

Back to **foot problems** or **foot pain** page

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Poor circulation (peripheral vascular disease)

Site Map

2:10
Edema

BRIEF COMMUNICATIONS

Lower-Extremity Edema Associated with Gene Transfer of Naked DNA Encoding Vascular Endothelial Growth Factor

Iris Baumgartner, MD; Guenter Rauh, MD; Ann Pieczek, RN; Debra Wuensch, RN; Meredith Magner, BA; Marianne Kearney, BS; Robert Schainfeld, DO; and Jeffrey M. Isner, MD

Background: Vascular endothelial growth factor (VEGF) promotes angiogenesis and vascular permeability. The extent to which VEGF may cause tissue edema in humans has not been established.

Objective: To evaluate patients undergoing VEGF gene transfer for evidence of lower-extremity edema.

Design: Prospective consecutive case series.

Setting: Hospital outpatient clinic.

Patients: 62 patients with critical limb ischemia and 28 patients with claudication.

Intervention: Gene transfer of VEGF DNA.

Measurements: Semiquantitative analysis of lower-extremity edema.

Results: Lower-extremity edema was observed in 31 of 90 (34%) patients. Edema was less common in patients with claudication than in those with pain at rest ($P = 0.016$) or ischemic ulcers ($P < 0.001$), and it was less common in patients with pain at rest than in those with ischemic ulcers ($P = 0.017$). Treatment was typically limited to a brief course of oral diuretics.

Conclusions: Vascular endothelial growth factor may enhance vascular permeability in humans. At the doses of plasmid DNA used in this study, lower-extremity edema responded to oral diuretic therapy and did not seem to be associated with serious sequelae.

Ann Intern Med. 2000;132:880-884.

For author affiliations, current addresses, and contributions, see end of text.

Vascular endothelial growth factor (VEGF) was discovered as a tumor-secreted factor that augments vascular permeability (1). The permeability-enhancing effect of VEGF has been estimated to be 50 times greater than that of histamine (2). After identification of the effects of VEGF on microvascular permeability, Leung (3) and Keck (4) and their colleagues demonstrated that VEGF may promote endothelial cell proliferation and migration. These findings led to preclinical animal studies (5–7) and human studies (8–10) that established that VEGF may promote angiogenesis in cases of limb ischemia.

We prospectively evaluated 90 patients with peripheral artery disease who underwent gene transfer of naked plasmid DNA encoding the 165–amino acid isoform of VEGF (phVEGF$_{165}$) for clinical evidence of enhanced vascular permeability. The primary results of these trials have been reported in preliminary form elsewhere (9–12).

Methods

Between December 1994 and July 1999, 90 patients (mean age ± SD, 59 ± 19 years) underwent gene transfer of phVEGF$_{165}$ as therapeutic angiogenesis (13) or to prevent restenosis after angioplasty (14). The protocols for these trials were approved by the institutional review board and institutional biosafety committee of St. Elizabeth's Medical Center, the Recombinant DNA Advisory Committee of the National Institutes of Health, and the Food and Drug Administration. Informed consent was obtained from all patients treated.

Intra-arterial gene transfer was performed in 40 patients. Of these patients, 28 with claudication underwent phVEGF$_{165}$ gene transfer after superficial femoral artery angioplasty; 4 patients with lower-extremity pain at rest and 8 patients with gangrene were treated to promote angiogenesis in the ischemic limb. Plasmid doses were 100 μg (1 patient), 500 μg (1 patient), 1000 μg (13 patients), 2000 μg (22 patients), and 4000 μg (3 patients).

Intramuscular gene transfer was performed in 50 patients, of whom 13 were treated for pain at rest and 37 presented with established gangrene. Five patients underwent treatment of the contralateral limb 3 or more months after treatment of the initial limb. Patients received 1000 μg ($n = 10$), 2000 μg ($n = 19$), 3000 μg ($n = 11$), and 4000 μg ($n = 10$) of phVEGF$_{165}$.

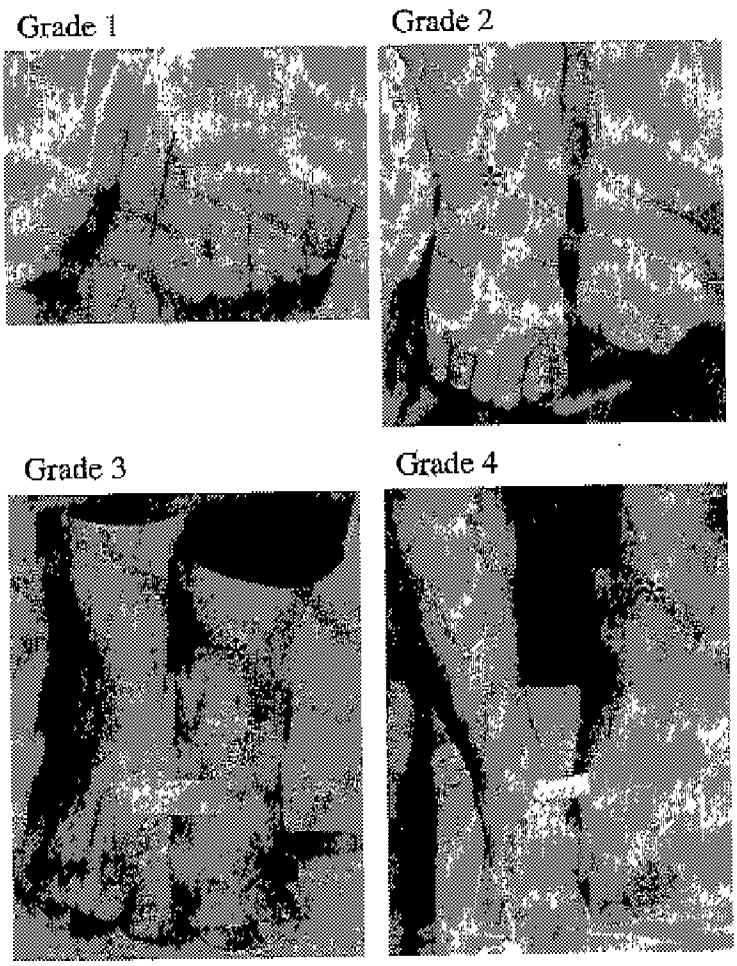

Figure. Representative examples of lower-extremity edema (*asterisks*) according to clinical grade in four patients who underwent intramuscular gene transfer of naked plasmid DNA encoding vascular endothelial growth factor. The extent and distribution of edema were unrelated to the sites of injection. For explanation of grades, see text.

Edema was scored jointly by two observers as follows: 0, no edema; 1, edema limited to the foot; 2, edema involving the foot and ankle; 3, edema involving the calf; 4, more than the three preceding symptoms (**Figure**).

Venous blood samples were analyzed by using enzyme-linked immunosorbent assay at baseline and weekly up to 4 weeks after the initial gene transfer, as described elsewhere (8).

Data are reported as the mean (±SE). The relation between nominal variables was calculated by using the Fisher exact test (2 × 2 contingency table). All statistical tests were two-tailed. A P value less than 0.05 indicated statistical significance.

Results

Development of Edema

Transient lower-extremity edema was observed after gene transfer of phVEGF$_{165}$ in 31 of 90 (34%) patients. Edema was graded as 1 in 9 patients, 2 in 13 patients, 3 in 6 patients, and 4 in 3 patients.

In 3 of 90 (3.3%) patients, edema developed in both limbs after gene transfer of phVEGF$_{165}$. All 3 patients had critical limb ischemia in both lower extremities.

Relation of Edema to Tissue Integrity

The incidence of peripheral edema differed among all subgroups. Peripheral edema developed significantly less frequently in patients without compromised tissue integrity than in patients with ischemic ulcers or gangrene. Peripheral edema associated with gene transfer was observed in 0 of 28 (0%) patients with claudication, 4 of 17 (24%) patients with pain at rest, and 27 of 45 (60%) patients with gangrene (**Table**). Results of the Fisher exact test showed that the incidence of edema differed significantly among all patient groups. Specifically, edema was less common in patients with claudica-

tion than in those with pain at rest ($P = 0.016$) or ischemic ulcers ($P < 0.001$) and was less common in patients with pain at rest than in those with ischemic ulcers ($P = 0.017$).

Intra-Arterial Compared with Intramuscular Gene Transfer

Peripheral edema was observed after both intra-arterial and intramuscular gene transfer (Table). After intra-arterial gene transfer, peripheral edema occurred in 0 of 28 (0%) patients with claudication, 1 of 4 (25%) patients with pain at rest, and 4 of 8 (50%) patients with established gangrene. After intramuscular gene transfer, peripheral edema occurred in 3 of 13 (23%) patients with pain at rest and 23 of 37 (62%) patients with gangrene. The difference between the incidence of lower-extremity edema after intra-arterial gene transfer (5 of 40 patients [12.5%]) compared with intramuscular gene transfer (26 of 55 patients [52%]) was statistically significant ($P < 0.001$) only when patients with claudication were included in the intra-arterial gene transfer group. When comparison of intra-arterial and intramuscular gene transfer was limited to patients in whom either method was used to treat pain at rest or gangrene, the incidence of edema did not differ significantly (5 of 12 patients [42%] compared with 26 of 50 patients [52%], respectively; $P > 0.2$). Development of edema was unrelated to the dose of $phVEGF_{165}$.

Time to Development of Edema

Clinically apparent peripheral edema usually developed within 3 weeks after intra-arterial or intramuscular gene transfer. Development of edema corresponded temporally to an increase in circulating levels of VEGF, consistent with the time course of gene expression (2 to 3 weeks) established for this plasmid in preclinical animal studies (7, 10). No differences were observed for intra-arterial compared with intramuscular gene transfer with regard to the time to development of edema or increased serum levels of VEGF.

Despite the temporal relation between the increase in serum VEGF levels and development of peripheral edema, no correlation was observed between the absolute peak level of VEGF and the appearance of peripheral edema. Similarly, no relation was seen between the absolute or relative increase in serum VEGF levels from baseline to peak levels and the presence or absence of edema.

Neither the magnitude (scores of 1 to 4) nor the probability of lower-extremity edema could be predicted by an individual VEGF level.

Recurrent Edema, Treatment, and Hospitalization

Development of peripheral edema was usually consistent among patients treated more than once. All patients who developed edema after the first gene transfer developed edema on subsequent injections, whereas no patient in whom edema failed to develop after the first gene transfer experienced edema on repeated gene transfer.

Treatment was usually initiated on an outpatient basis and consisted of oral diuretics. Patients most commonly received furosemide, bumetanide, or hydrochlorothiazide or a combination of these agents. Edema was promptly attenuated after administration of diuretics and resolved completely within 2 to 4 weeks after initiation of therapy.

In five patients, diuretics were administered intravenously during hospital admission; in none of these five patients was edema the principal indication for hospitalization. Four of the five patients were admitted for initiation of antibiotic treatment for suspected osteomyelitis, and one patient was admitted for control of ischemic pain at rest.

Vascular Endothelial Growth Factor–Enhanced Vascular Permeability and Evidence of Angiogenesis

Evidence of augmented collateral vessel development, including an increase in the ankle–brachial or toe–brachial index to a value greater than 0.1, newly visible collateral vessels on follow-up serial angiography, or marked improvement of ischemic gangrene and disappearance of ischemic rest pain, was seen in 43 of the 57 patients treated for critical limb ischemia. The relation between development of

Table. Frequency of Peripheral Edema after Intra-Arterial and Intramuscular Administration of Naked Plasmid DNA Encoding Vascular Endothelial Growth Factor

Status	Intra-Arterial Gene Transfer			Intramuscular Gene Transfer*	
	Patients with Claudication ($n = 28$)	Patients with Pain at Rest ($n = 4$)	Patients with Gangrene ($n = 8$)	Patients with Pain at Rest ($n = 13$)	Patients with Gangrene ($n = 37$)
	← n (%) →				
Edema	0	1 (25)	4 (50)	3 (23)	23 (62)
No edema	28 (100)	3 (75)	4 (50)	10 (77)	14 (38)

* No patient with claudication received intramuscular gene transfer therapy.

edema and evidence of enhanced angiogenesis was not statistically significant ($P > 0.2$).

Discussion

Our findings show that VEGF may augment vascular permeability in humans. The fact that edema failed to develop in patients with claudication but was observed in nearly half of the patients with resting ischemia suggests that the permeability-enhancing effects of VEGF are directly or indirectly potentiated by tissue ischemia.

Ischemic damage to the integrity of the microcirculation may directly potentiate the effect of VEGF. Similar loss of microcirculatory integrity in patients with critical limb ischemia frequently leads to edema after conventional revascularization; in that setting, successful bypass surgery leads to augmented blood flow, which, when superimposed on a damaged endothelial substrate, allows excess fluid transudation and, subsequently, development of clinically apparent edema. The potent vasodilating effects of VEGF, previously shown to be a potent stimulus for release of nitric oxide (15), together with vasoactive metabolites released from ischemic tissues may compound this effect. These mechanisms may have contributed to the development of bilateral edema in three patients with critical limb ischemia involving both lower extremities. In the absence of ischemia at rest, however, even the potent permeability-enhancing effects of VEGF were insufficient to cause clinically apparent edema.

Systemic administration of VEGF in a rabbit model of hind-limb ischemia was shown to cause selective neovascularization of the ischemic but not normally vascularized areas of the limb (5, 16). The localized biological effect observed in these experimental models, in which angiogenesis was selectively targeted to hypoxic tissues, seems to result from up-regulation of endothelial-cell VEGF receptor expression (17). This idea may explain the localization of clinically apparent edema to critically ischemic limbs.

The cellular and molecular regulation of VEGF-induced vascular permeability is incompletely defined. Dvorak and colleagues (2) found that vascular leakage could not be attributed to passage of molecules through inter–endothelial cell junctions or injured tumor endothelium but instead involved transendothelial transport by means of a novel cytoplasmic organelle that they termed the vesicular–vacuolar organelle. Other investigators (18) reported VEGF-induced ultrastructural features consistent with endothelial fenestration. More recently, we observed that VEGF-induced vascular permeability is attenuated by nitric oxide synthase inhibitors, the cyclooxygenase inhibitor indomethacin, and the tyrosine kinase inhibitor genistein (19).

From St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts.

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Fortunately the excitement of the war had diminished the number of those with imaginary diseases, but nonetheless he had found himself running repeatedly to his medical encyclopaedia in order to find out how to cope without all those things upon which he had always relied. He had memorized it from front to back with even more enthusiasm and dedication than a Muslim learns the Koran in order to become a Hafiz. Even so, his memory of it had by now diminished somewhat, since he had only ever had to employ parts of it regularly and had come to the realisation that most afflictions pass away by themselves, regardless of anything that he might do. Mostly it was a question of turning up and looking suitably solemn whilst performing the rituals of inspection.

Louis de Bernières
Corelli's Mandolin
New York: Vintage Books: 1994:120

Submitted by:
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Vascular endothelial growth factor-induced angiogenic gene therapy in patients with peripheral artery disease

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Abbreviations: ABI, ankle-brachial index; ASO, arteriosclerosis obliterans; CT, compulerized tomography; IE, immediate-early; PAD, peripheral artery disease; PEG, polyethylene glycol; TBI, toe-brachial index; VAS, visual analog scale

Abstract

This phase 1 clinical trial tested the safety of intramuscular gene transfer by using naked plasmid DNA encoding the gene for VEGF, and analyzed the potential therapeutic benefits in patients with severe peripheral arterial disease (PAD). This study was an open-labeled, dose-escalating, single-center trial on nine male patients with severe debilitating PAD who had not responded to conventional therapy. Seven had Buerger's disease and two had arteriosclerosis obliterans. Plasmid DNA (pCK) containing human *VEGF*$_{165}$ was given by eight intramuscular injections in and around the area in need of new blood vessels. The study evaluated three escalating total doses (2, 4, and 8 mg of pCK-*VEGF*$_{165}$), with half of each total dose given four weeks apart. The follow-up duration was nine months. The gene injections were well tolerated without significant side effects or laboratory abnormalities related to gene transfer. Three patients showed transient edema in their extremities. Ischemic pain of the affected limb was relieved or improved markedly in six of seven patients. Ischemic ulcers healed or improved in four of six patients. The mean ankle-brachial index (ABI) improved significantly. Six of nine patients showed an increase in collateral vessels around the injection sites demonstrated by digital subtraction angiography. However, there was no relationship between the degree of ABI improvement and the dose given. Mean plasma levels of VEGF did not increase significantly. In conclusion, intramuscular injections of pCK-VEGF$_{165}$ can be performed safely to induce therapeutic angiogenesis in patients with severe PAD.

Keywords: angiogenesis; gene therapy; peripheral vascular disease; vascular endothelial growth factor

Introduction

The age-adjusted prevalence of human peripheral artery disease (PAD) is approximately 12% (Criqui *et al.*, 1985). Symptoms of PAD include pain on walking (claudication) or at rest, and ulcers and gangrene in distal limbs. The most common causes of PAD are arteriosclerosis obliterans (ASO) and Buerger's disease (thromboangiitis obliterans; TAO). Despite advances in the treatment of PAD, many patients cannot be managed adequately with either medical therapy or revascularization procedures (Beard, 2000; Hiatt, 2001).

Therapeutic angiogenesis has emerged as a promising investigational strategy for the treatment of patients with PAD (Baffour *et al.*, 1992; Pu *et al.*, 1993; Takeshita *et al.*, 1994a, b). After more than a decade of preclinical studies and recent clinical trials, gene therapy has been established as a potential method for inducing therapeutic angiogenesis in patients with ischemic limb disease (Isner *et al.*, 1996; Baumgartner *et al.*, 1998; Isner *et al.*, 1998; Rajago-

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palan *et al.*, 2001; Shyu *et al.*, 2003).

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF) is specifically mitogenic for endothelial cells (Keck *et al.*, 1989; Leung *et al.*, 1989; Plouet *et al.*, 1989). This is an important advantage of VEGF for gene therapy because endothelial cells are responsible for neovascularization. Among the vectors used for clinical trials in angiogenic gene therapy, naked DNA is probably the safest and the most convenient vector. However, its approach has been limited because of low levels of gene expression in target tissues (Verma and Somia, 1997; Jeong *et al.*, 2002).

To develop a high-efficiency expression plasmid for naked *VEGF* DNA gene therapy, we have constructed an expression vector, pCK, that is able to drive high levels of gene expression in skeletal muscles (Lee *et al.*, 2000). A study of the use of pCK-$VEGF_{165}$ in an ischemic hind limb model using rabbits showed that it significantly increased distal limb blood flow (Chea *et al.*, 2001). We therefore performed a phase 1 clinical trial to evaluate the safety and potential efficacy of intramuscular gene transfer of pCK-$VEGF_{165}$ in Korean patients with PAD.

Materials and Methods

Patients

We included patients who had suffered chronic limb ischemia, including severe claudication, resting pain or nonhealing ischemic wounds (ulcers, gangrene) for a minimum of four weeks without evidence of improvement in response to conventional therapies, and who were not optimal candidates for surgical or percutaneous revascularization. Requisite hemodynamic deficits included a resting ankle-brachial index (ABI) of less than 0.6 and/or a toe-brachial index (TBI) of less than 0.3 in the affected limb on two consecutive examinations performed at least one week apart. All patients had angiographic evidence of superficial femoral artery or infrapopliteal disease in the affected limb. Criteria used to describe limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and the International Society for Cardiovascular Surgery (Rutherford and Becker, 1991). We excluded patients with a history of malignancy, proliferative diabetic retinopathy, a history of alcohol or drug abuse, or any other significant medical condition. All patients were allowed to continue previous medication. The study was designed as a phase I, nonrandomized, dose-escalating study to document the safety of intramuscular pCK-$VEGF_{165}$ gene transfer and to monitor clinical effects. This study protocol was approved by the Human Institutional Review Board, by the Institutional Biosafety Committee of Samsung Medical Center, and by the Korean Food and Drug Administration. This is the clinical trial of the first human gene therapy approved by the Korean Food and Drug Administration. All patients gave written informed consent before participation.

Screening tests before gene therapy

All patients had undertaken screening tests prior to gene injection. Possible malignancies were excluded by blood tests, urinalysis, stool examination, tumor markers, chest X-ray, chest computerized tomography (CT), abdominal ultrasonography, abdomen and pelvis CT, and by gastric and colonic fiberscopy. These tests were repeated at the end of study (nine months after the initial gene injection) to exclude malignancy. Fundoscopic examinations were performed before and at the end of the study.

Plasmid vector

pCK, the expression vector into which the $VEGF_{165}$ was inserted, has been described previously (Lee *et al.*, 2000). pCK contains not only the full-length immediate-early (IE) promoter of the human cytomegalovirus but also its entire 5' untranslated region upstream from the start codon of the IE gene. The plasmid DNA was produced to clinical grade according to a proprietary process established at Dong-A Pharmaceutical Co. (Seoul, Korea). Briefly, *E. coli* DH5α cells carrying the plasmids were grown in kanamycin-containing medium in a 15-l fermenter. The fermentation broth was subjected to a series of purification steps including centrifugation, alkaline lysis, ammonium acetate and polyethylene glycol (PEG) precipitation, anion exchange chromatography and gel filtration chromatography. The quality of purified plasmid was verified according to quality-control methods for clinical grade plasmid DNA. Finally, purified plasmid DNA was dissolved in saline (0.9% NaCl) at a concentration of 1 mg/ml and then dispensed into sterile vials.

Intramuscular injection regimen

The study evaluated an escalating set of total doses (2, 4, and 8 mg of pCK-$VEGF_{165}$) with half of each total dose given four weeks apart. Three patients were included for each total dose, and there was a three-month safety-check period before starting the next dose level in a new group of patients. Each dose was diluted with sterile saline to make the total volume 16 ml. A 2 ml aliquot of this was injected intramuscularly over two min at each site. We performed eight intramuscular injections using 23 gauge needles to the affected ischemic limb, mainly the calf and

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thigh muscles, without using local anesthesia. After the injections, we observed the patients for 30 min to check for any immediate adverse reactions. Four weeks after the first gene injection, a second set of injections was given at the same sites.

Hemodynamic and angiographic assessment

Patients were followed on a weekly basis for the first three months after gene injection, and at three-month intervals thereafter, for a total follow-up of nine months after the first injection. Ischemic ulcers were documented by color photography. Shrinkage of the ulcer area to less than 20% of the baseline area was defined as an improvement. Improvement of resting pain was evaluated using a visual analog scale (VAS) of pain scores. Patient recorded their VAS every day and the mean VAS of the previous week was calculated at each visit. The amount of analgesics used was recorded by questionnaire. Resting ABI or TBI were calculated from the quotients of absolute ankle or toe pressure to brachial pressure, respectively. Digital subtraction angiography was performed within one week before, and three and nine months after the first gene injection. During angiographic follow up, we maintained the same amounts of contrast, the force of contrast injection and the position of the catheter tip. New collateral vessel formation was assessed at the time at which contrast flow in the main conducting arteries was most clearly visible. New collateral vessels were assessed as +0 (no collateral development), +1 (slight), +2 (moderate), or +3 (rich) (Tateishi-Yuyama *et al.*, 2002).

Plasma VEGF concentrations

Plasma VEGF concentrations were measured at baseline and weekly up to 12 weeks after the initial gene injection. Samples were collected in sodium citrate tubes and immediately centrifuged for 20 min at 3,600 rpm at 4°C, and the plasma was stored at -20°C until analysis.

Plasma VEGF concentration was determined using an ELISA assay as follows. Human recombinant VEGF (Calbiochem, La Jolla, CA) was separated from the carrier protein by SDS-PAGE under reducing conditions and recovered by electroelution. The recovered VEGF was dialyzed against 50 mM carbonate buffer (pH 9.0), and the concentration of VEGF was adjusted to 0.5 μg/ml. Ninety six-well plates were coated with VEGF by incubating the plates with 100 μl/well of the VEGF solution for 16 h at 4°C. The VEGF solution was aspirated and the wells were blocked by incubating with 250 μl/well of 1% BSA in PBS for 1 h at room temperature. The wells were washed once with 1% sucrose and dried. The wells were incubated with 100 μl/well of patient plasma (diluted 1:10 in 30% BSA/PBS) for 1 h at 37°C. The wells were then washed five times with 0.05% Tween-20 in PBS, and bound antibody was detected by incubating the wells with 100 μl/well of anti-human IgG-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL), (diluted 1:10,000 in 30% BSA/PBS) for 30 min at 37°C. After washing five times with 0.05% Tween-20/PBS, 100 μl/well of TMB solution was added for color development and allowed to react for 30 min in the dark. The color reaction was stopped by adding 100 μl/well of 1 M H_2SO_4. Light absorbance at 450 nm was read using a microwell plate reader (Tecan, Switzerland).

Detection of anti-VEGF antibody

Plasma antibodies to VEGF were detected by immunoblotting at baseline and weekly up to 12 weeks, and at six and nine months after the initial gene injection. Recombinant human VEGF (Calbiochem) was separated from the carrier protein by SDS-PAGE under reducing conditions and transferred from the gel onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA) by electroblotting. After washing with PBS, reactive sites on the nitrocellulose membranes were blocked by incubating the strips in 3% BSA/PBS for 1 h at room temperature. The membranes were washed with PBS, dried and cut into strips 4 mm wide. The amount of VEGF immobilized on each strip was 0.1 μg. After three washes in PBS, the strips were incubated in 2 ml of plasma from patients (diluted 1:100 in 1% BSA/PBS) for 1 h with gentle shaking to allow the anti-VEGF antibodies in the plasma to bind to the immobilized VEGF band. The strips were washed three times with PBS, and immune complexes were detected with a 1:5,000 dilution of anti-human IgG-alkaline phosphatase (Calbiochem). After washing three times with PBS, the strips were developed in BCIP/NBT for 30 min to visualize bound antibody. The reaction was stopped by washing the strips with 20 mM EDTA/PBS.

Statistical analysis

Data are presented as means ± SEMs. Comparisons between variables from baseline to three or nine months were performed using nonparametric paired *t*-tests. Two-way ANOVA was applied for the comparison of mean plasma levels of VEGF. All statistical tests were two-tailed with significance assumed at $P < 0.05$.

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Results

Baseline clinical characteristics

All nine patients were male with a mean age of 51.0 ±14.9 years. There were seven TAO patients and two with ASO. Demographic and clinical data of the patients are shown in Table 1.

Safety assessment

There was mild discomfort up to 72 h after the in-

Table 1. Clinical, hemodynamic, and angiographic findings before and after gene therapy.

	Clinical history and findings before gene therapy						Outcomes after gene therapy	
No.	Age (years)	Total dose	Dx	Class	Previous treatment	Signs/ Symptoms	ABI (0/3/9 months) Limb status at 9 months	New collateral vessels (+3 to 0) (3/9 months)
1	40	2 mg	TAO	6	Sympathectomy Amputation	Claudication, Resting pain, Ulcer, Toe gangrene (digit III)	0.94/1.21/0.96 Resting pain improved Ulcer stationary	+2/+1
2	46	2 mg	TAO	5	Sympathectomy Amputation	Claudication Resting pain Ulcer (digit I) Toe gangrene (digit III)	0.5/1.09/0.97 Resting pain improved Ulcer improved	+2/+1
3	51	2 mg	TAO	5	Sympathectomy Amputation Beraprost	Claudication Resting pain Ulcer (digit I)	0.65/0.70/0.63 Resting pain and ulcer improved after bypass at six months	+2/+1
4	58	4 mg	TAO	5	Sympathectomy Amputation	Claudication Resting pain Ulcer (digit I) Toe gangrene (digit III, V)	0.40/0.58/0.64 Resting pain improved Ulcer improved	+3/+2
5	38	4 mg	TAO	5	1 Bypass Beraprost	Claudication Resting pain Ulcer (digit I)	0.57/0.60/0.70 Resting pain stationary Ulcer stationary	0/+1
6	72	4 mg	ASO	4	None	Resting pain	0.53/0.47/0.52 Resting pain improved	+1/0
7	41	8 mg	TAO	5	None	Claudication Resting pain Ulcer (digit I, V)	0.52/0.53/0.56 Resting pain improved Ulcer completely healed	0/0
8	76	8 mg	ASO	3	3 PTA, 1 bypass 2 Revision Beraprost	Claudication	0.00/0.31/0.38 Claucation improved	0/0
9	43	8 mg	TAO	5	Beraprost	Claudication Resting pain Ulcer (digit I)	0.59/0.88/0.93 Resting pain improved Ulcer completely healed	+2/+2

Dx, diagnosis; TAO, thromboangiitis obliterans; ASO, arteriosclerosis obliterans; Class, limb status adapted standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery (Rutherford *et al.*, 1991); PTA, percutaneous transluminal angioplasty.

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jections; however, gene injection was well tolerated without the need for local anesthesia. During the trial, no patient developed significant laboratory abnormalities in tests including those for complete blood counts, electrolyte concentrations, chemistry and lipid profiles, tumor markers, and inflammatory markers. No changes in diabetic retinopathy were observed from fundoscopic examinations. Likewise, no development of a latent neoplasm has been observed. Three patients complained of transient lower extremity edema, consistent with VEGF enhancement of vascular permeability, but this was completely controlled by diuretic therapy. Sensorineuronal hearing loss was observed in one patient (number 6 in Table 1), however this was judged as being unrelated to the gene transfer, and it resolved spontaneously. This event was reported to Human Institutional Review Board of our Institute and the Korean Food and Drug Administration, which approved continuation of the study.

Change in ischemic limb status

Therapeutic benefit was demonstrated in most of the patients by regression of resting pain and/or improved tissue integrity in the ischemic limb. A 51-year-old man (patient number 3), who had undergone below-knee amputation of the contralateral limb presented with severe resting pain and a necrotic great toe. He had focal stenosis of the distal femoral artery and occlusion of the infrapopliteal arteries. Even though angiographic findings at three months after gene therapy showed improved collateral vessels in the calf area, the ulcer had failed to respond. He had undergone short distal bypass of a stenotic lesion of the distal femoral artery using a graft from the great saphenous vein six months after gene injections. After surgery, his ulcer improved. This patient was thus excluded from the final assessment and eight patients were evaluated for clinical signs at the end of the study protocol.

Limb pain

Ischemic pain in the affected limb was markedly decreased after therapy. The VAS was 51.5 ± 9.6 mm at baseline ($n = 8$); 23.8 ± 9.7 mm ($n = 8$) at a three- month follow-up and 25.1 ± 9.5 mm ($n = 7$) at a nine-month follow-up ($P < 0.05$) (Figure 1A). Analgesic use per week showed a similar decline (data not shown). This improvement in resting pain was distinct in the first three months, and it was maintained until nine months. In two patients, however, the amount of analgesics could not be reduced. A 58-year-old man (patient number 4) presented with resting pain and ulcers on his toes and fingers. After gene transfer into the affected leg, while the resting pain of his foot decreased from a VAS of 74 at baseline to 20 at three months, the resting pain in his fingers did not improve, and he could not decrease his analgesic dose. A 38-year-old man (patient number 5) had continued resting pain and also could not decrease his analgesic intake.

Ischemic ulcers

Seven patients had ischemic wounds (ulcer and/or gangrene). Patient number 3 was excluded from the final assessment. Two patients with ischemic ulcers were completely healed after gene transfer and two more showed improvements. Representative photos are shown in Figure 2. Patients who had relatively larger ulcers experienced more difficulty in healing than the patients with smaller ulcers. In two patients (numbers 1 and 2), the lesions had progressed rapidly before therapy. However, this progression slowed down after gene transfer. Before gene transfer, seven

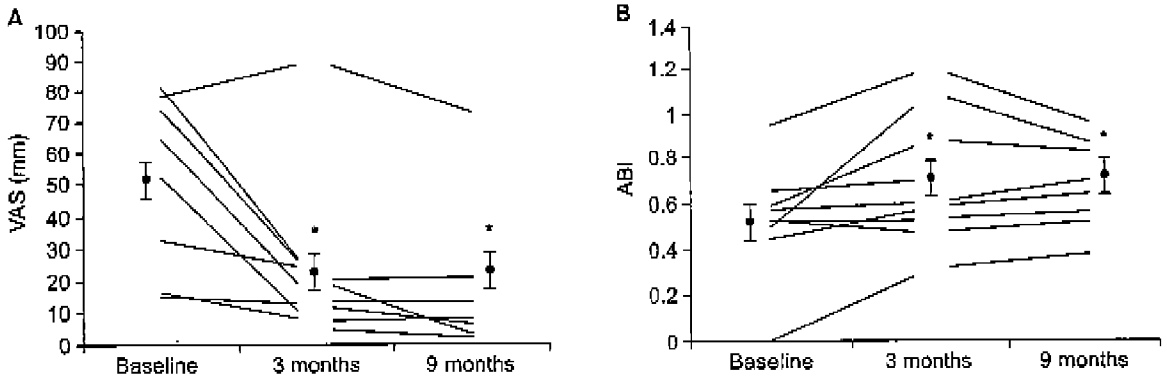

Figure 1. (A) Improvements in resting pain after gene therapy. VAS; visual analog scale. (B) Gain in ankle-brachial index (ABI) after gene therapy. The dot and bar indicate mean ± SEM. *$P < 0.05$ versus baseline levels.

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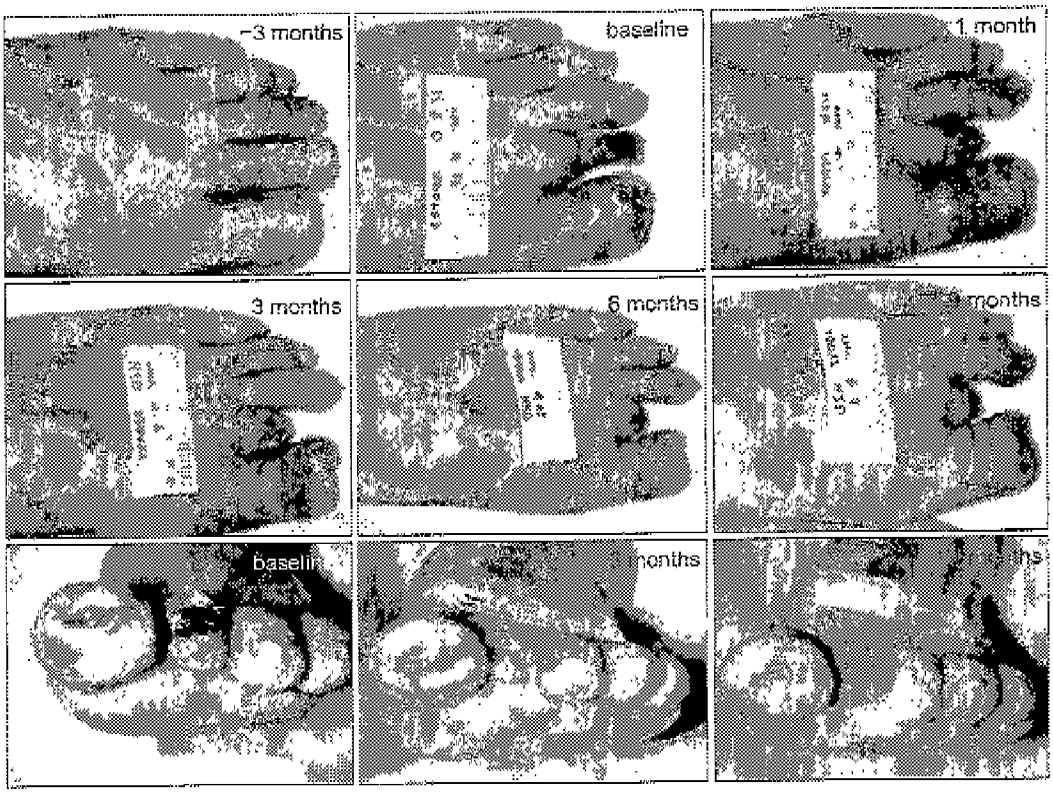

Figure 2. Limb salvage after gene therapy. Nonhealing ischemic wound of the great and second toes had been progressing rapidly before gene therapy. One month after gene injection, a second toe was self-amputated and its stump healed without ulcer progression. A nonhealing ulcer of the great toe also showed improvement after gene therapy. Before gene therapy, the patient was wheelchair bound and taking multiple analgesics. Nine months after gene transfer, he was freely walking and was free from resting pain after being successfully weaned from all analgesics.

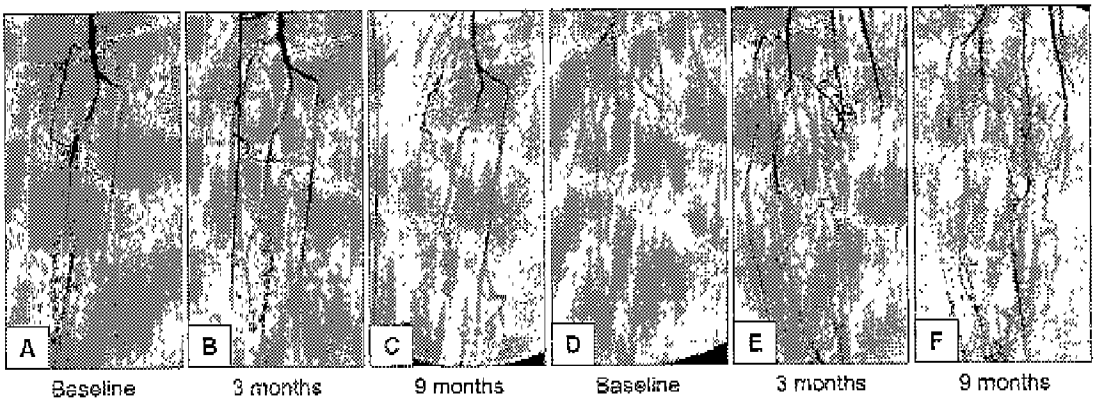

Figure 3. Subtraction angiographic analysis of collateral vessel formation. A, B and C from patient number 2. D, E and F from patient number 9. Compared with baseline angiography, newly developed collateral vessels were visible at the calf level three months after gene therapy. Increased collateral vessels remained at the nine-month follow-up. In patient number 2, semiquantitative analysis of new collateral vessels decreased from +2 at 3 month (3B) to +1 at 9 month (3C) after gene injection. In patient number 9, there was no regression of new collaterals.

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patients had been identified as candidates for limb amputation. After the therapy, three patients no longer needed amputation. Two patients avoided major amputation but underwent toe amputation after the nine-month follow-up. Seven of the eight patients showed significant improvements in resting pain and/or the conditions of their ischemic wounds at nine months.

Change in the ankle-brachial index

The ABI increased from 0.52 ± 0.08 (n = 9) at baseline to 0.71 ± 0.10 (n = 9) at three months (P = 0.025), and 0.69 ± 0.07 (n = 8) at nine months (P = 0.016) (Figure 1B). However, the extent of improvement was not correlated with the dose used ($P >$ 0.05). Improvement in the pressure index was sustained for up to nine months.

Angiographic findings

Three months after the beginning of gene transfer, lower extremity angiography showed newly visible collateral vessels at the knee, calf, and ankle level in six of the nine patients. Representative angiographs are shown in Figure 3. Contrast densities were similar suggesting that these were obtained under identical imaging conditions. The mean score of development of collateral vessels increased to 1.3 ± 0.4 at three months after commencing gene transfer ($P < 0.05$, n = 9) (Table 1). The newly developed collateral vessels persisted until nine months (mean score 0.9 ± 0.3, n = 8).

Blood levels of VEGF and VEGF antibody

Mean blood concentrations of VEGF did not increase from baseline level (9.8 ± 1.8 pg/ml). The measured amounts of VEGF after the initial gene injection were 8.0 ± 1.1, 6.5 ± 0.9, 8.4 ± 0.9, 7.6 ± 1.1, and 9.3 ± 1.7 pg/ml at 2, 4, 6, 8, and 12 weeks, respectively. No patient developed antibodies to VEGF.

Discussion

We have shown here that VEGF-induced angiogenic gene therapy is safe for human use and potentially effective as evidenced by substantial increases in ABI and by formation of new collateral vessel formation demonstrated on angiograms. pCK-$VEGF_{165}$ gene transfer not only improved resting pain in most of the patients, but also ischemic ulcers or gangrene in more than half of them.

The prognosis and quality of life for patients with chronic severe leg ischemia, as manifested by severe claudication, resting pain or ischemic ulcers, are poor. Patients who are not indicated for conventional revascularization therapy, called 'no-option patients', represent 10~15% of all PAD patients and usually receive only conservative management such as pain control and vasodilators (Wolfe 1986; Albers *et al.*, 1992). However, no pharmacological treatment has been shown to improve the natural history of severe limb ischemia (Isner and Rosenfield, 1993). Consequently, alternative treatment strategies are needed for these patients.

Collateral vessels are important as an alternative source of blood flow in the case of arterial stenosis or occlusion. They reduce limb ischemia and greatly improve the clinical symptoms such as claudication, resting pain, ischemic wounds and prognosis. Therapeutic angiogenesis is a new treatment strategy to increase collateral vessels in ischemic area by the injection of angiogenic factor or genes. VEGF is a specific angiogenic protein for endothelial cells (Keck *et al.*, 1989; Leung *et al.*, 1989; Plouet *et al.*, 1989). VEGF is induced by ischemia, and it induces endothelial cell proliferation and mobilization. Many experimental and clinical studies have reported that VEGF could be used as a method of treatment for patients with critical limb ischemia by the increase of the collateral vessel in ischemic areas (Takeshita *et al.*, 1994a, b; Isner *et al.*, 1996; Baumgartner *et al.*, 1998; Isner *et al.*, 1998; Shyu *et al.*, 2003).

Previous studies showed that intramuscular gene therapy with plasmid encoding human $VEGF_{165}$ ($phVEGF_{165}$) was safe and effective in the treatment of patients with critical limb ischemia (Baumgartner *et al.*, 1998; Isner *et al.*, 1998; Shyu *et al.*, 2003). In this phase I clinical trial, patients who received pCK-$VEGF_{165}$ gene transfers also showed improvements in resting pain and increases in collateral vessel growth. The resting pain improvements after gene transfer were more dramatic than the improvements in ABI or ischemic wounds. We speculate that the decrease in ischemic resting pain was caused not only by increased blood flow but also by recovery from ischemic neuropathy. Studies in animals show that constitutive over-expression of VEGF results in the restoration of large- and small-fiber peripheral nerve function in diabetic and ischemic neuropathy models (Schratzberger *et al.*, 2000; Schratzberger *et al.*, 2001; Veves and King, 2001). Preliminary clinical studies have also demonstrated improvement in signs and symptoms of sensory neuropathy in patients with PAD following intramuscular injection of $phVEGF_{165}$ (Isner *et al.*, 2001; Simovic *et al.*, 2001).

In previous studies with $phVEGF_{165}$, Baumgartner *et al.* and Shyu *et al.* reported that the gains in ABI scores three months after gene therapy were 0.15 and 0.14, respectively (Baumgartner *et al.*, 1998; Shyu *et al.*, 2003). In our study, the gain was 0.19; thus, even though the patient characteristics in these

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trials differed, all trials showed similar ABI score improvements. In the study of Shyu *et al.*, there appeared to be a dose-related response in terms of ABI and the minimal effective dose was a total of 2,400 μg (Shyu *et al.*, 2003). In our study, however, we could not find a dose response in terms of the incidence of edema, or improvements in resting pain, ABI or collateral development. In our previous dose-response study (Lee *et al.*, 2000), when pCK-*VEGF*$_{165}$ was injected into the tibialis anterior muscle of Balb/C mice, the level of local gene expression in the muscle reached a plateau at a dose of 125 μg, which corresponds to the dose injected at one site when using a total of 2 mg of plasmid in two sets of injections in this clinical trial. This could explain why we could not obtain a further gain at the higher dose rates. Thus, multiple injections with low doses (*i.e.* 125 μg/site) would be more effective than a few injections of high doses.

Another concern in our study is that we could not detect elevated levels of blood VEGF after gene transfer. Shyu *et al.* reported that mean plasma levels of VEGF increased significantly from a baseline of 26 ±31 pg/ml to 63±56 pg/ml two weeks after gene therapy (Shyu *et al.*, 2003). However, Freedman *et al.* (2002) reported that baseline plasma VEGF was highly variable and was not normally distributed in a study of the kinetics of VEGF protein release into the systemic circulation after gene transfer for 34 patients with PAD. In their study, after intramuscular gene transfer, median plasma VEGF rose slightly, although significantly, by seven days (38 to 41 pg/ml, $P < 0.05$), but was not different from baseline at 14, 21, or 28 days, and day-7 plasma levels did not differ significantly as a function of dose despite an almost 10-fold difference. They concluded that interpretation of results for individual subjects is complicated by wide variations in baseline VEGF and low circulating levels compared with baseline after gene transfer. Our data are in line with the above observations that baseline plasma VEGF concentrations are highly variable and there would be a marginal or no increase after intramuscular transfer of pCK-*VEGF*$_{165}$.

Finally, current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring < 200 μm in diameter (Takeshita *et al.*, 1997). Using synchrotron radiation microangiography, neovascularization after VEGF gene transfer in a rat model of hindlimb ischemia included a substantial contribution of vessels < 180 μm in diameter (Takeshita *et al.*, 1997). Thus, conventional angiographic techniques used in the present study may have failed to depict the full extent of angiogenesis achieved after pCK-*VEGF*$_{165}$. In this sense, Laser doppler imaging (LDI) used to detect cutaneous blood flow and/or tissue oxymetry (TcO_2) may give an additional clue to assess the improved perfusion in treated limbs.

Our study is a phase I clinical trial. Therefore, the safety of patients is a major aim of the study and it is not designed to draw conclusions regarding efficacy. However, clinical efficacy including resolution of resting pain, and healing of ischemic ulcers was associated with objective findings of improved ABI and blood flow on angiography.

In conclusion, this phase I, open-label, dose-escalating study showed that intramuscular *VEGF* gene transfer was safe and feasible in Korean patients with chronic severe leg ischemia. The procedure was well tolerated without complications directly related to gene expression except for transient leg edema. Even though the procedure was associated with clinical improvements, phase II and III trials will help determine the efficacy of this therapy in patients with severe PAD.

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Clinical Investigation and Reports

Constitutive Expression of phVEGF$_{165}$ After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

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Background—Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries, a concept called "therapeutic angiogenesis." The objectives of this phase 1 clinical trial were (1) to document the safety and feasibility of intramuscular gene transfer by use of naked plasmid DNA encoding an endothelial cell mitogen and (2) to analyze potential therapeutic benefits in patients with critical limb ischemia.

Methods and Results—Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers (n=7/10) and/or rest pain (n=10/10) due to peripheral arterial disease. A total dose of 4000 μg of naked plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF$_{165}$) was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly (0.33±0.05 to 0.48±0.03, P=.02); newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Tissue specimens obtained from an amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells by immunohistochemistry. PCR and Southern blot analyses indicated persistence of small amounts of plasmid DNA. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability.

Conclusions—These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. (*Circulation*. 1998;97:1114-1123.)

Key Words: angiogenesis ■ genes ■ ischemia ■ growth substances

Critical limb ischemia is estimated to develop in ≈500 to 1000 individuals per million per year.[1] In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization, and the disease thus frequently follows an inexorable downhill course.[2,3] Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with cancer in the terminal phase of their illness.[4] As concluded in the Consensus Document of the European Working Group on Critical Limb Ischemia,[1] no pharmacological treatment has been shown to favorably affect the natural history of critical limb ischemia.[5] Indeed, amputation, despite its associated morbidity, mortality, and functional implications,[1,6–8] is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain, of critical limb ischemia.[9–12] A second major amputation will be required in nearly 10% of such patients. Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

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Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral[13,14] and myocardial[15–17] ischemia, a concept called therapeutic angiogenesis. Several of these studies have used VEGF, also known as vascular permeability factor, a secreted endothelial-cell mitogen with high-affinity binding sites limited to endothelial cells.[18–22] Endothelial cell specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.[23–25]

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Selected Abbreviations and Acronyms

ABI = ankle-brachial index
MRA = magnetic resonance angiography
PCR = polymerase chain reaction
$phVEGF_{165}$ = plasmid encoding 165-amino-acid isoform of human VEGF
TBI = toe-brachial index
VEGF = vascular endothelial growth factor

We recently demonstrated angiographic and histological evidence of angiogenesis after intra-arterial gene transfer of naked plasmid DNA encoding human VEGF in a patient with critical limb ischemia.[26] In this report, we present the results of intramuscular $phVEGF_{165}$ gene transfer performed in an initial phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.

Methods

Patients

Patients qualified for intramuscular gene therapy if they (1) had chronic critical limb ischemia[1] including rest pain and/or nonhealing ischemic ulcers present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies and (2) were not optimal candidates for surgical or percutaneous revascularization.[27] Requisite hemodynamic deficit included a resting ABI <0.6 and/or TBI <0.3 in the affected limb on 2 consecutive examinations performed at least 1 week apart. Criteria used to describe a change in limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery.[3,28] Patients were allowed to continue on aspirin and coumarin, provided that these therapies had been used for a minimum of 6 months before gene transfer. Vasoactive medications were discontinued unless prescribed for cardiac disease or systemic hypertension. All patients gave written informed consent for their participation. The study was designed as a phase 1, nonrandomized study to document the safety of intramuscular $phVEGF_{165}$ gene transfer and to monitor patients as well for evidence of bioactivity. This study design was unanimously approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, by the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth's Medical Center, and by the US Food and Drug Administration.

Plasmid DNA ($phVEGF_{165}$)

All patients received a eukaryotic expression vector encoding the $VEGF_{165}$ gene[29] transcriptionally regulated by the cytomegalovirus promoter/enhancer.[26] Preparation and purification of the plasmid from cultures of $phVEGF_{165}$-transformed *Escherichia coli* were performed in the Human Gene Therapy Laboratory at St Elizabeth's Medical Center by the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses.

Intramuscular $phVEGF_{165}$ Transfer

Aliquots of 500 μg of $VEGF_{165}$ pDNA were diluted in sterile saline, and 4 aliquots (total, 2000 μg) were administered into calf and/or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The volume of each of the 4 injectates per limb was progressively increased during the course of the study from 0.75 mL (3 treatments) to 3 mL (6 treatments) to 5 mL (11 treatments). Four weeks after the first 2000-μg injection, a second 2000-μg injection was administered, increasing the total amount of pDNA to 4000 μg per patient. One patient was treated for bilateral critical limb ischemia with a total amount of 8000 μg pDNA (4000 μg per limb).

Serum VEGF Levels

ELISAs were performed at baseline and weekly up to 12 weeks after the initial treatment of 7 limbs to detect evidence of gene expression at the protein level. Samples were immediately centrifuged for 20 minutes at 3600 rpm at 4°C, and the serum was stored at −20°C until analysis. Serum VEGF was determined with an immunoassay according to the manufacturer's instructions (R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate.

Hemodynamic and Angiographic Assessment

Patients were followed up on a weekly basis within the first 8 weeks after gene therapy and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Resting ABI and TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure.[30] Intra-arterial digital subtraction angiography and MRA were performed within 1 week before and 4 weeks after each treatment and 3 months after the latter of 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study using undiluted nonionic contrast media (Isovue-370, Squibb Diagnostics). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and ankle/foot levels were recorded by digital acquisition and hard copies in a 35×45-cm format. The diameter of newly visible collateral vessels was assessed by comparison with a 0.09-in-diameter reference wire taped to the skin. MRA was performed with a 1.0-T superconducting system (Impact, Siemens) by means of a transmit-receive extremity coil, a body coil, or both and commercially available pulse sequences. A multisection two-dimensional time-of-flight gradient echo sequence without intravenous contrast medium was used.[31] All axial images were reconstructed by use of the maximum-pixel-intensity algorithm at intervals of 60°.

Immunohistochemistry

Double immunohistochemical staining for proliferating endothelial cells was performed as previously described.[32] Bound antibody was then detected with an alkaline phosphatase kit (Biogenex Laboratories). Complexes were visualized with fast red substrate (Biogenex Laboratories). A counterstain of 10% Gill hematoxylin was applied before coverslips were applied.

DNA Analysis

Skin specimens at the site of gene injection and muscle specimens near or remote from the site of gene injection were retrieved from 2 amputees 8 and 10 weeks after intramuscular $phVEGF_{165}$ transfer, respectively (patients 4 and 10, Table). Tissue was processed with a genomic DNA isolation kit (A.S.A.P., Boehringer Mannheim). For PCR analysis, primer sets unique to the promoter and VEGF coding region of $phVEGF_{165}$ were selected. For Southern analysis, *Eco*RI-digested total cellular DNA (30 μg) and purified $phVEGF_{165}$ DNA (0.5 μg) were subjected to 0.8% agarose electrophoresis. The predicted sizes of *Eco*RI-digested plasmid fragments were 998 and 4703 bp. DNA blotted to a nylon membrane (Amersham, Life Science) was hybridized with two ^{32}P-labeled $phVEGF_{165}$-specific probes (*ncol*-digested 679-bp $phVEGF_{165}$ fragment, position 389 to 1068; *aval*-digested 787-bp $phVEGF_{165}$ fragment, position 991 to 1778), washed, and exposed to Hyperfilm MP (Amersham, Life Science).

Statistical Analysis

Data are reported as mean±SEM. Comparisons between paired variables were performed with the nonparametric Friedman test and Wilcoxon rank sum test. All statistical tests were two-tailed, with a significance level of $P<.05$.

Clinical, Hemodynamic, Angiographic, Laboratory, and Molecular Findings Before and After Intramuscular phVEGF$_{165}$ Gene Transfer

			Clinical History and Findings Before Gene Therapy				Outcome After Gene Transfer	
No.	Sex	Age, y	Cigs, pk/y	DM	Previous Treatment	Signs/Symptoms	Limb Status	DSA Findings
1	F	33†	30	0	4 bypass grafts, 3 rev., prostaglandins	Calf ulcer, toe gangrene (digit I)	ABI +0.24; complete healing→limb salvage	New collaterals, 200–400 μm
2	F	53	0	+	3 bypass-grafts, 1 PTA, prostaglandins	Toe gangrene (digit V)	ABI +0.12; complete healing	New collaterals, 200–400 μm
3	M	77	0	+	None	Toe gangrene (digits I, IV)	TBI +0.11; gangrene/ osteomyelitis→BKA	New collaterals, 200–400 μm
4	F	39†	20	0	Sympathectomy	Forefoot gangrene	ABI +0.27; forefoot necrosis→BKA	New collaterals, 200–400 μm
5	M	74	90	0	1 PTA	Rest pain	ABI +0.15; rest pain resolved	New collaterals, 200–800 μm
6	F	84	40	0	6 bypass grafts, 1 PTA	Toe gangrene (digits I–V)	ABI +0.22; toe amputation→limb salvage	None
7	F	80	20	0	1 bypass graft	Rest pain	ABI unchanged, rest pain resolved	New collaterals, 200–800 μm
8*	F	39	20	0	Sympathectomy	Heel ulcer, toe gangrene (digits I–V)	ABI +0.22; toe amputation→limb salvage	New collaterals, 200–>800 μm
9	M	54	30	0	4 bypass grafts, 2 rev., 1 PTA	Rest pain	TBI +0.18; rest pain resolved	None
10	M	54	70	0	6 bypass-grafts, 1 PTA	Toe gangrene (digits I, III, IV)	No change in ABI/TBI, BKA	None

No. indicates consecutively treated ischemic limbs; Cigs, current cigarette smoker; pk/y, pack years of cigarette smoking; DM, diabetes mellitus (non–insulin-dependent DM, oral medication); DSA, digital subtraction angiography; BI, baseline; rev., surgical revisions; PTA, percutaneous transluminal angioplasty; TBI (ABI incompressible); BKA, below-knee amputation; ND, not done; and pos., positive.

*No. 8 and 4 identical patient (bilateral treatment).

†Suspected Buerger's disease (stopped smoking >3 months before study entry).

Results

Demographic and clinical data for the 5 women and 4 men (mean age, 59±19 years) treated with phVEGF$_{165}$ are shown in the Table. Average length of follow-up at the time of this report was 6±3 months (range, 2 to 11 months). Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial creatine phosphokinase measurements remained in the normal range, there were no signs of systemic or local inflammatory reactions, and no patient developed antibodies to VEGF. To date, neither loss of visual acuity nor change in funduscopic examination due to diabetic retinopathy[33] has been observed in any patient treated with phVEGF$_{165}$ gene transfer. Likewise, no development of a latent neoplasm[34] has been observed. The only complication seen was transient lower-extremity edema, consistent with VEGF enhancement of vascular permeability.[35]

Transgene Expression

Blood levels of VEGF transiently peaked 1 to 3 weeks after gene transfer in 7 patients in whom weekly blood samples were obtained (Fig 1). (In 2 patients, baseline and/or more than two follow-up blood samples were not obtained.) Indirect clinical evidence of VEGF overexpression was evident from the development of peripheral edema (+1 to +4 by gross inspection) in the 6 patients with ischemic ulcers. In 4 of these patients, edema was limited to the treated limb, whereas in 2 patients, the contralateral limb was affected as well, albeit less severely. The onset of edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive Arterial Testing

Absolute systolic ankle or toe pressure increased in 8 limbs after gene transfer and was unchanged in 1 limb at the time of the most recent follow-up (53±4.8 at baseline, 66±4.6 most recent follow-up, P=.008). ABI and/or TBI increased from 0.33±0.05 (range, 0 to 0.58; n=10) at baseline to 0.43±0.04 (0.22 to 0.57, P=.028; n=10) at 4 weeks, to 0.45±0.04 (0.27 to 0.59, P=.016; n=10) at 8 weeks, and to 0.48±0.03 (0.27 to 0.67, P=.017; n=8) at 12 weeks (Fig 2). Improvement in the pressure index was sustained, but the increases in values obtained after the second (4-week) injection were not significantly different from measurements made 4 weeks after the initial injection. Exercise performance improved in all 5 patients with rest pain or ischemic ulcers who were able to perform graded treadmill exercise.[36] All patients experienced a significant increase in pain-free walking time (2.5±1.1 minutes before gene therapy versus 3.8±1.5 minutes at an average of 13 weeks after gene therapy, P=.043). A statistically significant increase in absolute, claudication-limited

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Continued

VEGF Level, pg/mL			
BI	1st	2nd	Molecular Findings
47	223	607	
36	ND	ND	
46	131	780	
30	59	688	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens
62	300	96	
29	164	80	
40	44	223	
ND	ND	ND	
0	113	83	
ND	ND	ND	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens

walking time (4.2±2.1 minutes before versus 6.7±2.9 minutes after gene therapy, $P=.018$) was documented as well. Two patients reached the target end point of 10 minutes of exercise.

Angiography

Digital subtraction angiography showed newly visible collateral vessels at the knee, calf, and ankle levels in 7 of 10 treated ischemic limbs. The luminal diameter of the newly visible vessels ranged from 200 to >800 μm, although most were closer to 200 μm; the latter frequently appeared as a "blush" of innumerable collaterals (Fig 3A and 3B). Follow-up angiograms disclosed no evidence of collateral artery regression in any patients. Serial magnetic resonance angiograms of the ischemic limb disclosed qualitative evidence of improved distal blood flow in 8 limbs, including enhancement of signal intensity in previously identified vessels, and an increase in the number of newly visible collaterals (Fig 3C and 3D).

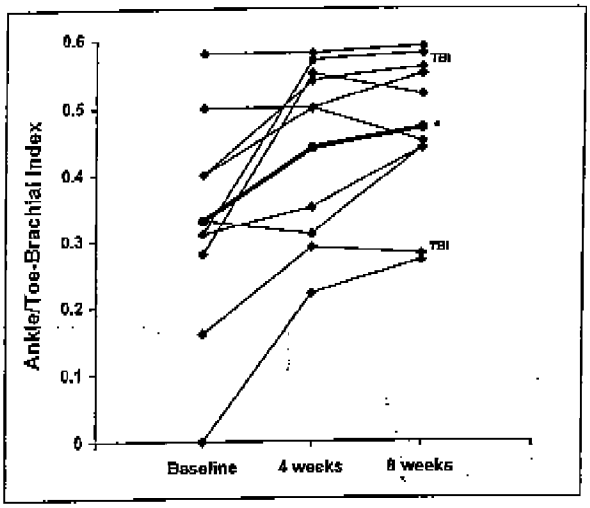

Figure 2. Gain in ABI and/or TBI in 10 limbs 4 and 8 weeks after intramuscular phVEGF$_{165}$ gene transfer. *Mean values, $P=.02$.

Change in Limb Status and Ischemic Rest Pain

Therapeutic benefit was demonstrated by regression of rest pain and/or improved tissue integrity in the ischemic limb. Limb salvage, for example, was achieved in a 33-year-old woman (patient 1, Table), who had undergone 7 unsuccessful surgical reconstructions at another hospital. She presented with a necrotic great toe and a 9×3-cm ischemic ulcer at the site of vein harvest in her distal left limb (Fig 4). The ulcer had failed to respond to 6 months of conservative measures, during the last 3 of which she had been treated with methadone, oxycodone/acetaminophen, amitriptyline hydrochloride, and a fentanyl patch. She had been advised by her vascular surgeons to undergo below-knee amputation. Within 8 weeks after gene transfer, her ABI had increased by 0.24, and the ulcer dimensions had diminished sufficiently to permit placement of a split-thickness skin graft. The graft healed successfully and remained healed at 9-month fol-

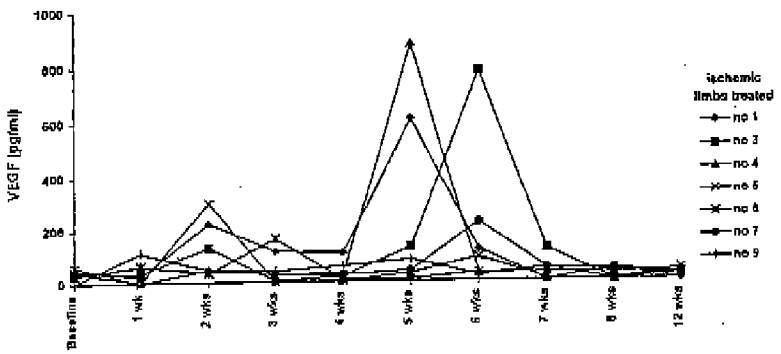

Figure 1. Serial levels of VEGF determined by ELISA disclosed a transient elevation 1 to 2 weeks after intramuscular (phVEGF$_{165}$) gene transfer. Baseline and/or weekly follow-up venous blood samples, which were incomplete in 3 of 10 treated limbs (patients 2, 8, and 10 in the Table), are not shown.

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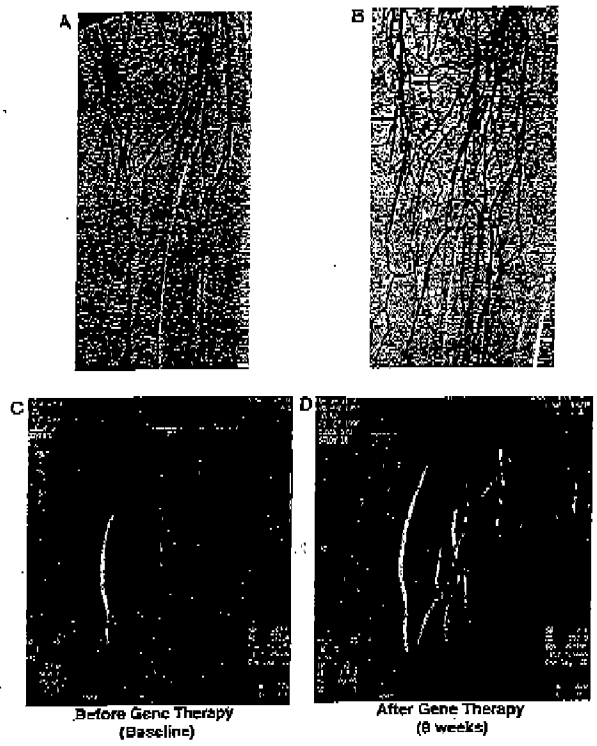

Figure 3. A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF$_{165}$ gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800 μm (arrow); most were closer to 200 μm, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

low-up (Fig 4). A second patient, a 39-year-old woman (patients 4 and 8, Table), presented with a 3-month history of gangrene of the distal half of her right foot. Although the ABI in her right limb improved by 0.27 after gene transfer, the forefoot gangrene was not reversed, and she underwent right below-knee amputation. While she was being treated for the right limb, however, she developed gangrene in the left limb (Fig 5). After gene transfer to the left limb, the ABI in her left lower extremity increased by 0.22 in association with angiographically demonstrable, newly visible collateral vessels. Although she required amputation of the left great toe, the operative site healed promptly, and her remaining 4 toes and heel recovered completely, including restoration of normal nail growth (Fig 5). In 2 other patients, complete (patient 2, Table) or partial (patient 6, Table) healing of ischemic ulcers present for 12 and 2 months, respectively, avoided major limb amputation. In the 3 patients (patients 5, 7, and 9, Table) who presented with rest pain (of 6, 5, and 3 months' duration, respectively) unassociated with loss of tissue integrity, rest pain resolved completely in all 3 patients after gene transfer and has remained so at the most recent follow-up.

For the total group of 10 limbs, frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly (5.9±2.1 at baseline versus 1.5±2.8 at 8-week follow-up, P=.043). On the basis of criteria proposed by Rutherford et al,[3] limb status improved in 9 of 10 extremities treated (Table). Moderate improvement, including both an upward shift in clinical category (≥1 clinical category in patients with rest pain and ≥2 categories in patients with tissue loss) and an increase of >0.1 in the ABI, was documented in 5 patients.

Immunohistochemistry and Molecular Analyses

Tissue specimens retrieved from 1 amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells (Fig 6A). This finding was particularly striking given that endothelial cell proliferation is nearly absent in normal arteries.[37] PCR performed on these samples indicated persistence and anatomic redistribution of DNA fragments unique to phVEGF$_{165}$. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the injection site (Fig 6B). Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimens derived from 2 amputees 8 and 10 weeks after gene therapy (patients 4 and 10, Table) (Fig 6C).

Discussion

The natural history of critical limb ischemia has been well documented to have an inexorable downhill course.[3] The inclusion criteria for this study were drafted to restrict treatment to patients in whom the natural history of critical limb ischemia had been established previously. Seven of the 10 limbs had developed frank gangrene. Although inclusion criteria required a minimum of 4 weeks of conservative measures without evidence of improvement, in reality, signs and/or symptoms of critical limb ischemia had been present in all cases for 2 to 12 months before gene therapy. Among this series of 9 patients (10 limbs), 6 developed critical limb ischemia despite having undergone as many as 7 vascular surgical reconstructions. Seven patients had been specifically advised to undergo limb amputation. All were using analgesic, typically ≥1 narcotic, medications. Spontaneous resolution of rest pain and/or healing of an ischemic ulcer in patients like these with critical limb ischemia has not to our knowledge been reported previously.[1] Furthermore, because VEGF had not been administered previously as recombinant protein, no data were available from any source to indicate either the safety or bioactivity of any dose of phVEGF$_{165}$. Accordingly, the design of this phase 1 trial, unanimously approved by the Recombinant DNA Advisory Committee and the US Food and Drug Administration, was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols used to study new chemotherapeutic agents administered to human subjects.

Analysis of gene expression at the protein level by use of an ELISA assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after gene transfer in 7 patients. Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema, including 2 with bilateral edema. Parenthetically, the latter finding constitutes what is to the best of our knowledge the first demonstration that VEGF may augment vascular permeability in human subjects.

In most patients, treatment was sufficient to achieve clinically significant modulation of the recipient phenotype.

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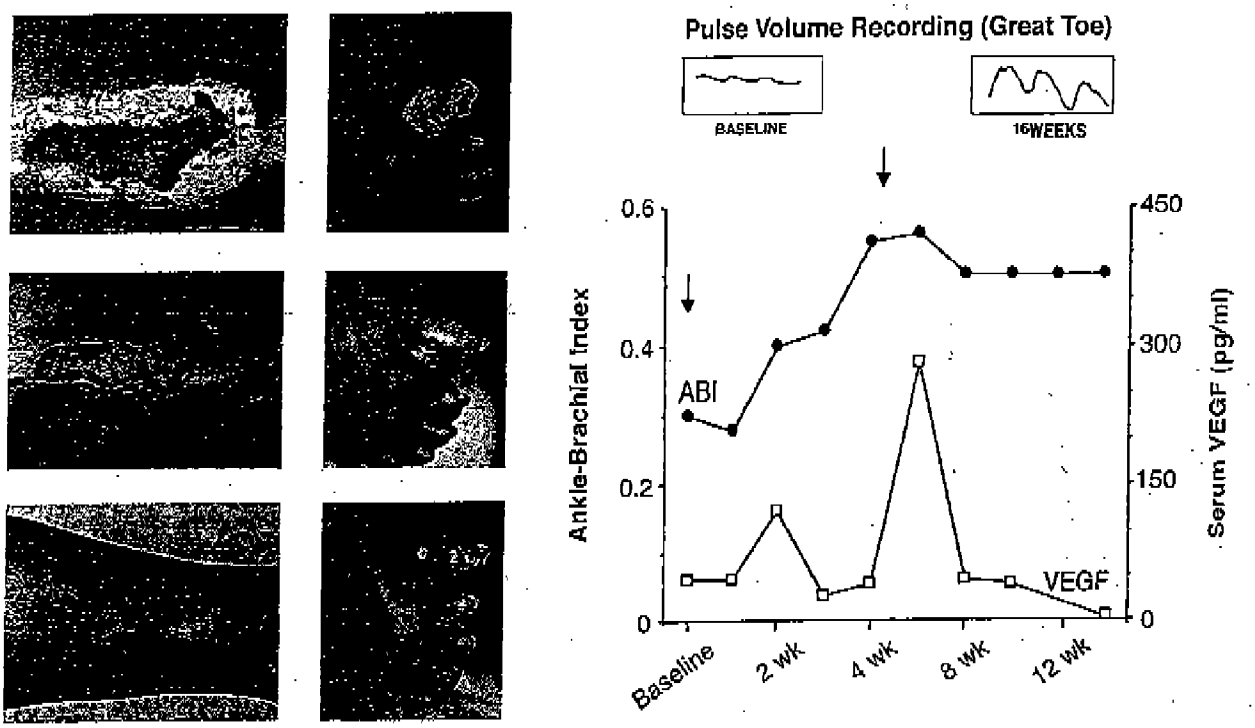

Figure 4. Limb salvage after gene therapy in a 33-year-old woman (patient 1, Table). Left top, Nonhealing wound on medial aspect of calf and ischemic necrosis involving great toe. Left middle, Ingrowth of granulation tissue in calf wound, healing of great toe. Left bottom, Three months after gene transfer, healing of split-thickness skin graft at wound site and full resolution of great toe necrosis. Before gene therapy, patient was wheelchair-bound on multiple analgesics, including methadone, amitriptyline hydrochloride, clonidine, oxycodone/acetaminophen, and a fentanyl patch. Three months after gene transfer, she was freely ambulatory and had been successfully weaned from all analgesics. Right, Evidence of phVEGF$_{165}$ bioavailability documented by an increase in venous VEGF blood levels and bioactivity expressed as an increase in ABI. The ABI progressively increased from 0.28 before to 0.56 after gene therapy (weeks refer to time after transfection). This was associated with development of a phasic pulse volume recording compared with nonphasic tracing recorded at baseline. Vertical arrows indicate timing of each of the 2 intramuscular phVEGF$_{165}$ injections.

Noninvasive studies documented hemodynamic evidence of improved limb perfusion that satisfies outcome criteria proposed to assess the results of surgical reconstruction or percutaneous revascularization.[28] Absolute ankle and/or toe pressure increased in 8 limbs after gene therapy (P=.008). ABI and/or TBI increased from 0.33±0.05 at baseline to 0.48±0.03 at 12 weeks (P=.017). To put this in perspective, an increase of >0.1 in the ABI is considered indicative of a successful surgical or percutaneous intervention.[28] To the best of our knowledge, such improvement has not previously been achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels, accompanied here by noninvasive (MRA) evidence of improved blood flow, has to the best of our knowledge not been reported previously in response to any therapeutic intervention. Indeed, previous reports have indicated that current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring <200 μm in diameter[38]; the spatial resolution of images obtained by MRA is even less. Using synchrotron radiation microangiography to assess collateral artery development after VEGF gene transfer in a rat model of hindlimb ischemia, Takeshita et al[38] showed that neovascularization included a substantial contribution of vessels <180 μm in diameter. Thus, conventional angiographic techniques used in the present study may have failed to depict the full extent of angiogenesis achieved after phVEGF$_{165}$ transfection, particularly given that most newly visible collaterals were diminutive (200 to 800 μm).

That angiogenesis was in fact therapeutic in the present investigation was shown by concomitant reduction in rest pain and/or a favorable impact on limb integrity. Rest pain resolved in all 3 of the patients who presented with rest pain alone. Ischemic ulcers present in 7 limbs healed or improved markedly in 4 patients; this included 3 patients recommended for below-knee amputation in whom successful limb salvage was achieved. Given the poor prognosis for patients with chronic critical limb ischemia, in whom the possibility of spontaneous improvement is remote,[1,2] the outcome in this initial cohort is thus encouraging.

Beginning with the reports of Wolff et al,[39] work from several laboratories[40–48] convincingly demonstrated evidence of transgene expression after direct injection of nonviral, covalently closed pDNA into skeletal muscle. The conceptual basis for therapeutic angiogenesis after phVEGF$_{165}$ gene transfer in particular has been established previously by our laboratory.[49,50] The results of the present trial extend previous findings from studies performed in live animals[46] to patients with advanced peripheral artery disease.

The failure of previous gene therapy trials to yield evidence of clinical success has been attributed to gene delivery,

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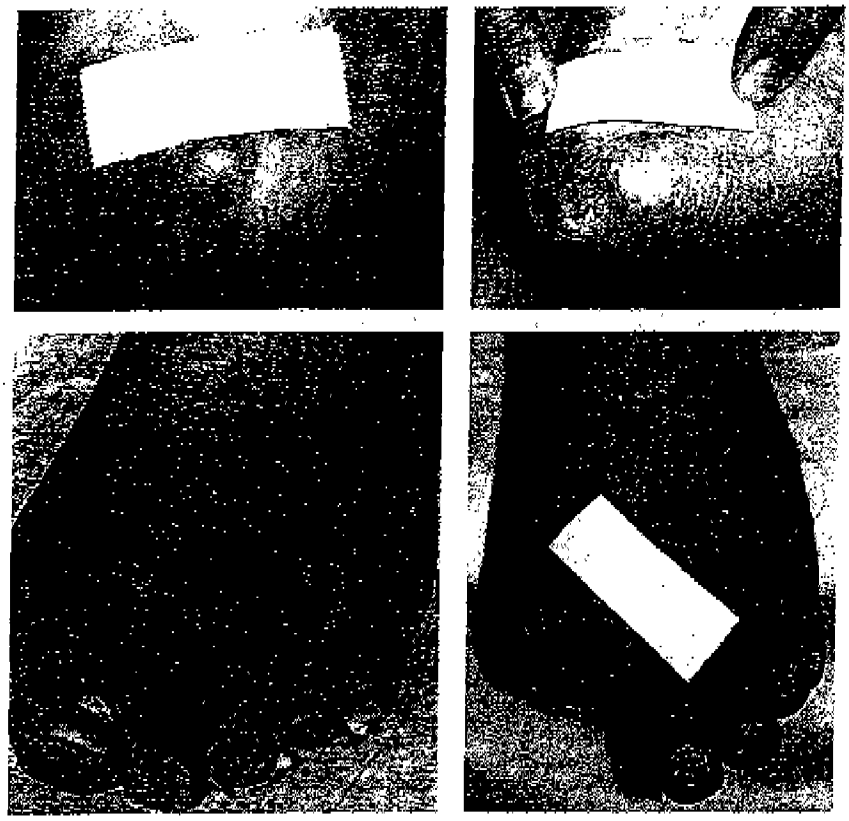

Figure 5. Limb salvage after gene therapy in a 39-year-old woman (patient 8 and 4, Table). This patient presented with a 3-month history of gangrene of distal one-half of right foot. Although ABI in right limb improved by 0.27, forefoot gangrene was not reversed, and she underwent below-knee amputation. While she was being treated for right limb, however, she developed gangrene of left great toe and shortly after, of 4 remaining left toes as well. After gene transfer to left limb, her ABI increased by 0.22 in association with angiographically demonstrable new collateral vessels. Although she required a great toe amputation, operative site healed promptly, and remaining 4 toes recovered completely, including restoration of normal nail growth.

specifically the inability to deliver genes efficiently and to obtain sustained expression.[51] Those cases in which phVEGF$_{165}$ gene therapy led to successful clinical outcomes in this clinical trial suggest that the success of gene therapy is not solely a function of transfection efficiency, nor is it necessarily dependent on protracted gene expression. Several aspects of the gene, protein, and target tissue may have contributed to successful modulation of the host phenotype, despite the relatively low transfection efficiency typically associated with naked DNA. First, VEGF, as noted above, is actively secreted by intact cells; previous studies in our laboratory[52] have documented that genes that encode for secreted proteins, as opposed to proteins that remain intracellular, may yield meaningful biological outcomes because of paracrine effects of the secreted gene product. Second, heparin avidity of the VEGF$_{165}$ isoform promotes binding to cell surface and matrix heparan sulfates that may create a biological reservoir of the secreted protein, enhancing the temporal opportunity for bioactivity. Third, although endothelial cells were previously viewed solely as the target for VEGF, it is now clear that endothelial cells subjected to hypoxia can synthesize VEGF as well.[53] This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, because endothelial cell proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Fourth, VEGF inhibits apoptosis,[54] in part by upregulating endothelial cell expression of fibronectin and $\alpha_v\beta_3$,[54,55] thus preserving the survival signal generated by attachment of endothelial cells to their extracellular matrix. Such reduction in endothelial cell apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net increase in endothelial cell viability. Fifth, with regard to the target of gene therapy, it has been noted[14,26,49] that VEGF-induced angiogenesis is not indiscriminate or widespread but rather is restricted to sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (*Kdr*) in response to factors released from hypoxic skeletal myocytes.[56] Receptor upregulation on endothelial cells within the region of lower-limb or myocardial ischemia thus enables these cells to act as magnets for any VEGF secreted into the ischemic milieu. Finally, the fact that the host tissues are by definition hypoxic may directly aid intramuscular transfer of naked DNA, because transfection efficiency is augmented when the injected skeletal muscle is ischemic.[40,46]

Previous work from our laboratory established that phVEGF$_{165}$ transgene expression is limited to <30 days in animal models of limb ischemia.[26,46,49] Although Southern blot and PCR analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from 2 amputees in this clinical trial, we have no evidence to suggest that transgene expression is more protracted in human subjects than in our animal models. Fortuitously, however, it appears that in both animals and humans, collateral vessel development sufficient to restore limb perfusion to satisfactory resting levels occurs within this time interval. Cessation of gene expression beyond this time point can be considered to constitute an inherent safety feature of phVEGF$_{165}$ gene

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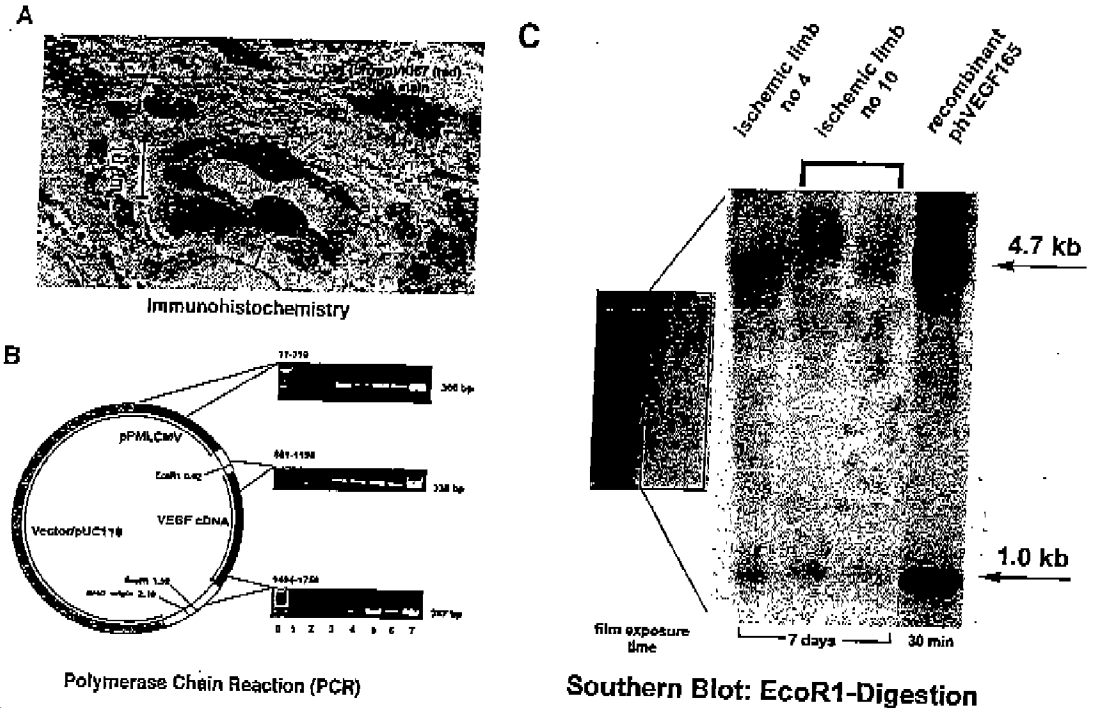

Figure 6. Immunohistochemical and molecular analyses of tissue specimens derived from 2 amputees 8 and 10 weeks after gene therapy (limbs 4 and 10, Table). A, Double immunohistochemical staining of tissue specimen with monoclonal antibody to CD31 (brown) and polyclonal antibody to Ki-67 (red) shows proliferating microvascular endothelial cells (arrows). B, PCR demonstrates amplification of $phVEGF_{165}$ DNA fragments in skin and skeletal muscle specimens. Lane 0 shows 100-bp ladder; lane 2, reaction mixture without tissue DNA; lane 3, negative control (skin specimen from untreated patient); lanes 4 to 6, specimens derived from $phVEGF_{165}$-treated amputee (patient 4, Table); lane 4, skin; lane 5, gastrocnemius muscle (remote from site of transfection); lane 6, tibialis anterior muscle (transfection site); lane 7, positive control (purified $phVEGF_{165}$). Amplified fragments had predicted sizes of 300, 338, and 257 bp, spanning the CMV promoter/enhancer region (5′-CCCGACATTGATTATTGA-3′ and 5′-CGGGCCATTTACCGTCAT-3′; position 11 to 300), proximal VEGF encoding region and junction between VEGF encoding region and promoter (5′-GCCTTTCTCTCCACAGGT-3′ and 5′-GTACTCGATCTCATCAGG-3′; position 861 to 1198), and distal VEGF encoding region and junction between VEGF encoding region and SV40 polyadenylation sequence (5′-CGCGTTGCAAGGCGAGGC-3′ and 5′-GGACCCAAAGTGCTCTGC-3′; position 1494 to 1750), respectively. C, Southern blot analysis of *Eco*R1-digested total cellular DNA (30 μg) (lane 1, patient 4, Table; lanes 2 and 3, patient 10, Table), and 0.5 μg (lane 4) of purified $phVEGF_{165}$ DNA hybridized with two ^{32}P-labeled $phVEGF_{165}$ specific probes (position 389 to 1068 and position 991 to 1778) showed persistence of complete plasmid DNA (*Eco*R1-digested 4703- and 998-bp fragments) in skeletal muscle specimens derived from 2 amputees (patients 4 and 10, Table).

transfer that protects the organism from indefinite constitutive expression of an angiogenic growth factor.

Several caveats regarding this preliminary clinical experience must be acknowledged. First, it is theoretically possible that VEGF expression resulting from gene transfer could promote the development of a tumor that is currently too small to be recognized. Previous laboratory studies, however, have established that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis, a finding in agreement with the notion that stimulation of angiogenesis is necessary but not sufficient for malignant growth.[36,57] It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy.[33] To date, however, no change in visual acuity has been observed in any patient treated with $phVEGF_{165}$ gene transfer. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Second, although it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, from the standpoints of both safety and efficacy, to a single larger dose of recombinant protein, this notion remains to be proven. Preliminary clinical trials of recombinant VEGF protein therapy have confirmed that mild hypotension seen in preclinical studies[15,58] may be seen in humans as well (unpublished data). Presumably, the route and/or dose of recombinant protein delivery can be adjusted to address this issue. Clearly, further clinical studies of both recombinant protein and alternative dosing regimens of gene therapy will be required to define the relative merits of each approach. Third, we cannot exclude the possibility that these encouraging preliminary results might have been made more substantial and/or uniform by the use of alternative vector systems and/or dosing strategies.[45,48,51,59]

In summary, these preliminary data may be cautiously interpreted to support both the strategy of intramuscular gene therapy and the concept of therapeutic angiogenesis for treatment of selected patients with critical limb ischemia.

Acknowledgments

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